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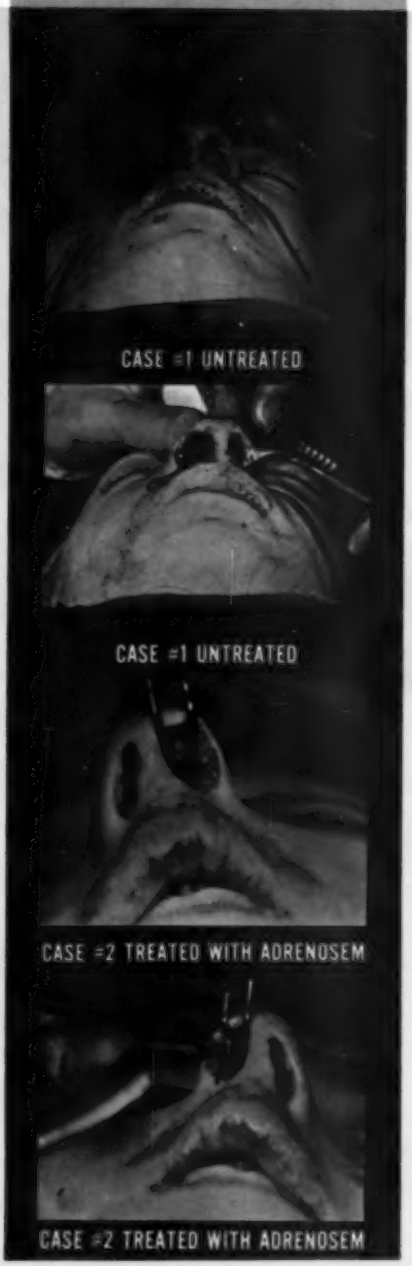

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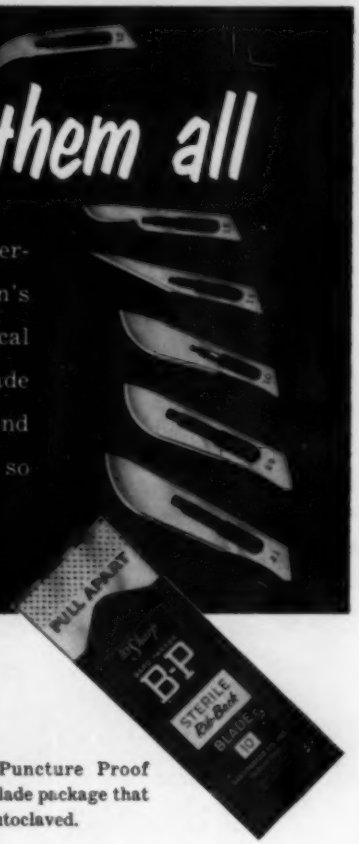

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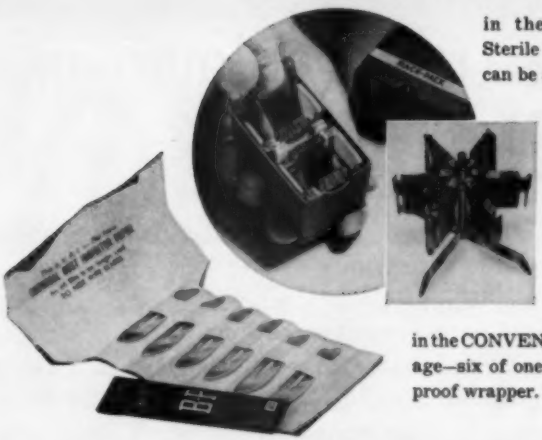

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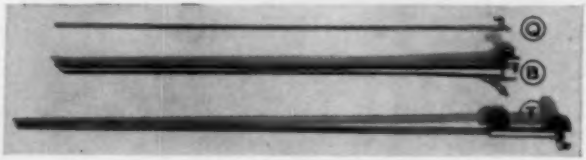

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Letter from the Editor

The Editor wishes to take this opportunity to express his appreciation to Dr. Charles A. LeMaistre, Dr. Richard L. Riley, Dr. David D. Rutstein, and Dr. Robert Shaw for their high quality of performance and diligent service as members of the Editoral Board.

New members of the Board as of July 1, 1958, are Dr. David T. Carr, Mrs. Shirley H. Ferebee, Dr. Edward A. Gaensler, and Dr. Paul C. Samson, who have accepted three-year appointments.

THE AMERICAN REVIEW OF TUBERCULOSIS AND PULMONARY DISEASES

VOLUME 78 *July, 1958* NUMBER 1

INTRAPULMONARY GAS MIXING FOLLOWING LUNG SURGERY[1,2]

With Particular Reference to Tuberculosis

EMIL BLAIR[3,4] AND JOHN B. HICKAM

(Received for publication January 31, 1958)

INTRODUCTION

Pulmonary tuberculosis may cause impairment of gas mixing within the lungs to the extent that a considerable volume of lung tissue is ineffectively ventilated (1). Because surgical removal of diseased lung is a common procedure in the therapy of tuberculosis, it is important to know how this is likely to affect intrapulmonary gas mixing. Significant further impairment would mean the functional loss of some lung tissue in addition to that removed at surgery. Previous studies of the effect of surgically removing lung tissue have shown that gas mixing, as measured by various methods, is not generally impaired (2–4). Except for occasional cases, however, the patients did not have tuberculosis, and the remaining lung was presumably free of significant disease. The results, therefore, need not necessarily apply to tuberculous patients, in whom the remaining lung may still be involved to some extent by a disorder which, in itself, can compromise gas mixing.

Recently, quantitative methods for the study of intrapulmonary gas mixing have been developed in several laboratories (5–8). It is the purpose of the present report to describe the application of one of these methods to the preoperative and postoperative study of a group of patients who have had extirpative pulmonary surgery, primarily for tuberculosis.

METHODS

Details of the method used for measurement of the functional residual capacity and the volume and ventilation rate of the slowest ventilated lung region ("slow space") have previously been described (8).

[1] From the Department of Medicine, Duke University School of Medicine, Durham, North Carolina.

[2] This work was supported by a grant (H-1370(CA)), from the National Heart Institute of the National Institutes of Health, Public Health Service.

[3] Formerly United States Public Health Service Post-Doctorate Research Fellow.

[4] Present address: The Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland.

TABLE 1

PULMONARY FUNCTION BEFORE AND AFTER LUNG SURGERY

Patient Number	Age	Sex	Surface Area M^2	Diagnosis	Operation	Pre- and Postoperative Periods	MBC		VC		FRC L	RC L	TC L	RC/TC × 100	MV L
							L/Min	Per Cent of Predicted	L	Per Cent of Predicted					
1	29	F	1.65	Tuberculosis, moderately advanced RUL	Lobectomy, RUL	Preoperative	101.4	107	4.65	127	3.21	1.24	5.99	23	10.4
						9 days	55.0	59	1.86	52	2.48	1.69	3.55	48	13.0
2	33	M	2.02	Tuberculosis, moderately advanced, bilateral	Lobectomy, LUL	Preoperative	149.5	107	3.91	91	3.80	2.83	6.74	42	11.7
						8 days	93.5	67	2.40	55	2.81	1.60	4.00	41	11.3
						8.5 months	112.0	80	3.28	76	2.64	1.37	4.65	29	12.7
3	29	F	1.62	Tuberculosis, moderately advanced, RLL	Lobectomy, RLL	Preoperative	98.8	106	2.95	95	2.33	0.83	3.78	22	12.4
						8 days	48.0	51	1.70	55	1.58	0.74	2.49	32	11.0
						8 months	103.0	111	1.37	44	2.41	1.55	3.22	57	13.3
4	36	M	1.88	Tuberculosis, far advanced, bilateral	Lobectomy, RUL	Preoperative	104.6	81	3.94	89	2.33	1.02	4.96	21	10.3
						11 days	68.9	54	2.07	47	1.06	0.68	2.55	19	9.9
						8 months	81.9	65	1.78	40	2.00	1.44	3.22	45	11.9
5	30	M	1.89	Lung abscess, RUL	Lobectomy, RUL	Preoperative	136.5	116	2.74	72	2.36	1.22	3.96	31	14.0
						12 days	87.2	74	1.76	46	1.81	1.35	3.11	43	12.9
						7.5 months	135.0	115	3.50	92	3.41	2.46	5.96	41	12.3
6	29	F	1.90	Pectus excavatum, bronchiectasis, bilateral	Lobectomy, RLL	Preoperative	92.2	89	2.99	86	2.76	1.66	4.65	36	11.8
						7 months	71.5	69	1.62	46	2.74	2.12	3.74	57	12.5

7	49	M	1.80	Tuberculosis, far advanced, RUL	Lobectomy, RUL	Preoperative	48.0	44	3.12	81	2.98	1.93	5.05	38	11.7
						6 months	85.0	59	2.75	71	2.85	2.23	4.98	45	17.0
8	30	M	1.64	Tuberculosis, far advanced, LUL	Lobectomy, LUL	Preoperative	79.2	68	2.05	50	1.97	1.25	3.30	38	9.8
						6 months	93.6	81	2.14	52	1.96	1.18	3.32	36	12.8
9	36	F	1.73	Tuberculosis, far advanced, bilateral	Lobectomy, RUL	Preoperative	40.7	43	1.70	56	2.24	1.49	3.19	47	10.0
						9 months	40.3	43	0.81	27	1.97	1.66	2.47	67	9.0
10	54	M	2.05	Tuberculosis, moderately advanced, RUL	Lobectomy, RUL	Preoperative	81.9	69	3.45	94	3.96	3.23	6.68	48	12.5
						11.5 months	67.6	58	1.62	47	4.09	3.49	5.11	68	12.0
11	40	M	2.00	Tuberculosis, far advanced, bilateral	Pneumonectomy, right	Preoperative	92.2	70	2.65	61	2.22	1.61	4.36	37	13.7
						7.5 months	65.0	50	3.04	70	2.79	1.64	4.68	35	10.4
12	41	F	1.44	Tuberculosis, far advanced, left	Pneumonectomy, left	Preoperative	48.2	64	1.43	54	1.17	0.84	2.27	37	7.7
						8.5 months	51.0	68	0.62	24	1.15	1.10	1.72	65	8.3
Mean and standard deviation						Preoperative	88 ± 35	78 ± 25	2.81 ± .82	75 ± 17	2.56 ± .80	1.63 ± .78	4.45 ± 1.39	36 ± 9	11.4 ± 2.1
						Late postoperative	82 ± 28	75 ± 23	2.05 ± .98	54 ± 21	2.55 ± .79	1.87 ± .69	3.92 ± 1.27	50 ± 14	12.0 ± 2.3

MBC: Maximal breathing capacity.
VC: Vital capacity.
FRC: Functional residual capacity.
RC: Residual capacity.
TC: Total capacity.
MV: Minute ventilation.
RUL: Right upper lobe.
LUL: Left upper lobe.
RLL: Right lower lobe.

Briefly, the subject first breathes a mixture of 50 per cent helium and 50 per cent oxygen for fifteen minutes. At the end of a quiet breath, he is switched to oxygen alone, and the expired gas is thereafter collected for measurement of the total amount of helium which can be washed out of the lungs. From this measurement the functional residual capacity is calculated, as in other open-circuit methods. In addition, the helium concentration in the expired gas is followed continuously by a katharometer between levels of 3.30 and 0.05 per cent helium. When this "wash-out" curve is plotted on semilogarithmic paper, the terminal portion usually falls along a straight line. The emergence of a straight line indicates that those portions of the lung which are still excreting helium are behaving like a single, homogeneously ventilated lung region, the "slow space."

By graphic analysis it is possible to estimate the volume and the ventilation rate of this slow space. The slow space may be described in terms of its relative size and its turnover rate. The relative size "f" is the ratio between the volume of the slow space and the functional residual capacity. The turnover rate "k" is the ratio between minute ventilation and the volume of the slow space. For technical reasons, it is not feasible by this method to estimate the volume and the ventilation rate of lung regions having a k greater than 1.0.

The vital capacity and its subdivisions and the maximal breathing capacity were measured with a spirometer in the conventional manner (9). The residual capacity was obtained by subtracting expiratory reserve volume from the functional residual capacity. Predicted values for vital capacity and maximal breathing capacity were calculated by formulas (10). All measurements were made with the subjects in the seated position.

Results

Twelve patients were studied, all but 2 of whom had tuberculosis. Studies were done preoperatively, in the early postoperative state (eight to twelve days) in 6 cases, and in the late postoperative period (five to eleven months) in 11 cases. Ten patients had lobectomies and 2 had pneumonectomies.

Data relative to age, disease, operation, and changes in lung volumes, maximal breathing capacity, and resting minute ventilation appear in table 1. Preoperatively, 6 of the 12 patients had a maximal breathing capacity of less than 80 per cent of the predicted normal, and in 5 cases the vital capacity was reduced below this level. There was considerable variation in residual capacity in the group, but in general this tended to be relatively large, as indicated by the ratio of residual lung volume to total capacity. In the normal young to middle-aged adult this ratio does not usually exceed 30 per cent, with slightly higher values for patients in the older age groups. Of the present subjects, 8 had ratios greater than 35 per cent in the preoperative period.

In the early postoperative period the maximal breathing capacity and the vital capacity were decreased below the preoperative levels in each case in which the measurements were made. In the late postoperative period there remained a significant decrease in vital capacity ($P < 0.025$), but the maximal breathing capacity was not significantly reduced below the preoperative value. The residual capacity (RC) tended to rise and the total capacity (TC) to fall in the late postoperative period, with the result that the RC/TC ratio was significantly increased over the preoperative level ($P < 0.025$).

Data relative to the most slowly ventilated lung spaces are presented in table 2. There is considerable variation in the extent to which gas mixing is affected preoperatively, as noted in a previous study of patients with tuberculosis (1), but

TABLE 2

CHARACTERISTICS OF THE POOREST VENTILATED SPACE (Vs) BEFORE AND AFTER LUNG SURGERY

Patient Number	Operation	Pre- and Postoperative Periods	k	f	$\dot{V}s$	Vs	Helium Clearance Time	f/k
			($\dot{V}s/Vs$)	(Vs/FRC)	ml/min	Liters	(minutes)	
1	Lobectomy, RUL	Preoperative	0.77	0.50	1230	1.60	6.5	0.65
		9 days	1.38				4.3	
2	Lobectomy, LUL	Preoperative	0.60	0.50	1340	2.24	8.1	1.00
		8 days	0.58	0.33	800	0.85	6.5	0.65
		8.5 months	0.55	0.42	610	1.10	6.8	0.76
3	Lobectomy, RLL	Preoperative	0.46	0.11	120	0.25	5.0	0.24
		8 days	0.99	0.44	600	0.69	4.5	0.44
		8 months	0.28	0.12	100	0.29	6.5	0.43
4	Lobectomy, RUL	Preoperative	0.69	0.43	700	1.00	7.0	0.62
		11 days	1.73				3.7	
		8 months	1.26				4.0	
5	Lobectomy, RUL	Preoperative	1.07				4.7	
		12 days	0.60	0.32	350	0.58	5.0	0.53
		7.5 months	0.56	0.48	680	1.19	6.7	0.86
6	Lobectomy, RLL	Preoperative	0.69	0.15	280	0.41	6.0	0.22
		7 months	0.46	0.15	190	0.46	5.8	0.33
7	Lobectomy, RUL	Preoperative	0.32	0.36	340	1.06	11.0	0.89
		6 months	0.30	0.29	230	0.83	8.2	1.00
8	Lobectomy, LUL	Preoperative	0.42	0.43	360	0.86	8.0	1.00
		6 months	0.46	0.33	290	0.64	7.0	0.72
9	Lobectomy, RUL	Preoperative	0.32	0.48	340	1.08	11.7	1.50
		9 months	0.27	0.47	250	0.93	12.5	1.74
10	Lobectomy, RUL	Preoperative	0.19	0.18	130	0.65	12.0	1.00
		11.5 months	0.29	0.46	570	1.90	12.2	1.59
11	Pneumonectomy, right	Preoperative	0.50	0.44	550	1.10	7.0	1.00
		7.5 months	0.40	0.47	520	1.30	9.7	1.17
12	Pneumonectomy, left	Preoperative	0.53	0.31	190	0.36	6.3	0.59
		8.5 months	0.41	0.37	170	0.43	7.0	0.90
Mean and standard deviation		Preoperative	0.53±.24	0.34±.17*	410±380*	0.89±.60*	7.9±2.6	0.83±.41*
		Late postoperative	0.48±.28	0.34±.12	330±190	0.88±.48	7.9±2.6	0.96±.48

* Mean values exclude No. 4 and No. 5, in whom the slow space could not be measured.

k: Turnover rate of slow space (minutes $^{-1}$).

f: Fraction which the slow space constitutes of the resting lung volume.

$\dot{V}s$: Ventilation rate of slow space (ml/minute).

Vs: Volume of slow space (liters).

Helium clearance time: The time in minutes required for the concentration of helium in the expired gas to fall to 1/1,000 of the starting value.

RUL: Right upper lobe.

LUL: Left upper lobe.

RLL: Right lower lobe.

the group as a whole showed significant impairment in both preoperative and postoperative periods.

A group of 29 normal subjects studied in this laboratory, and having the same average age (thirty-seven years) as the present subjects, had a mean k, or turnover rate of the slow pace of 0.94, S.D. $\pm$ 0.29, and a helium clearance time (*see* table 2) of 5.9 minutes, S.D. $\pm$ 1.5. The normal turnover rate is significantly faster ($P < 0.01$) and the clearance time shorter ($P < 0.01$) than the corresponding values for the present subjects, both before and after operation. The normal

f/k ratio rarely reaches 1.00 (1), but this level was attained or exceeded by several of the present patients before or after operation.

In comparing the late postoperative with the preoperative state, there were no significant differences in the mean values for size or turnover rate of the slow spaces, for the f/k ratio, or for the helium clearance time. As a group these patients showed impaired intrapulmonary gas mixing of about the same degree before and after surgery.

Discussion

In these patients, removal of lung tissue caused a significant loss of vital capacity and a significant increase in the ratio of residual lung volume to total capacity. These changes after extirpative lung surgery have been well documented elsewhere (2–4). In nontuberculous patients gas mixing has been found to be normal after extirpative lung surgery. The present patients showed impairment of gas mixing of about the same moderate degree before and after surgery. It is encouraging to some extent that surgery did not cause a marked further impairment in this aspect of pulmonary function. The results, however, suggest that surgery may not be entirely benign with respect to its effect on gas mixing. It is likely that gas mixing was particularly defective in the most severely diseased portions of the lung, which were removed at surgery. The finding that mixing is equally defective after operation suggests that a considerable portion of the remaining lung is less well ventilated in the postoperative state than it was before surgery.

Summary

A study has been made of intrapulmonary gas mixing before and after lobectomy or pneumonectomy in 12 patients, 10 of whom had moderately advanced or far advanced tuberculosis.

As a group, these patients showed moderate impairment of intrapulmonary gas mixing both before and after surgery. The failure of gas mixing to improve following removal of the most severely diseased portion of the lung suggests that a considerable part of the remaining lung may be less well ventilated than it was before surgery.

Sumario

La Mezcla Intrapulmonar de Gases Consecutivamente a la Cirugía Pulmonar: Con Referencia Particular a la Tuberculosis

Este estudio versa sobre la mezcla intrapulmonar de gases antes y después de la lobectomía o la neumonectomía en 12 enfermos, 10 de los cuales tenían tuberculosis moderadamente avanzada o muy avanzada.

Como grupo, estos enfermos revelaron moderado empeoramiento de la mezcla intrapulmonar de gases tanto antes como después de la cirugía. La falta de mejoramiento de la mezcla de gases después de extirpar la porción más enferma del pulmón sugiere que una considerable parte del pulmón restante tal vez quede menos bien aireada de lo que estaba antes de la operación.

Resume

Mélange des gaz intrapulmonaires à la suite de la chirurgie pulmonaire: particulièrement en ce qui concerne la tuberculose

Une étude du mélange gazeux intrapulmonaire avant et après lobectomie ou pneumonectomie a été effectuée chez 12 malades dont 10 présentaient une tuberculose modérément ou très avancée.

Dans l'ensemble, ces malades présentaient une altération modérée du mélange des gaz aussie bien avant qu'après l'intervention chirurgicale. Le défaut d'amélioration du mélange gazeux, à la suite de la résection des segments pulmonaires les plus atteints, suggère qu'une partie considérable du parenchyme pulmonaire restant pourrait être moins bien ventilé qu'il ne l'était avant l'intervention chirurgicale.

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PLEURAL BIOPSY IN DIAGNOSIS OF EFFUSIONS[1,2]

Results In Fifty Cases of Pleural Disease Observed Consecutively

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INTRODUCTION

Thorough study of patients with pleural disease, utilizing traditional diagnostic techniques, frequently fails to yield a specific diagnosis. It has been shown by Roper and Waring (1) that, if young adults with pleural effusion and positive tuberculin skin tests are returned to normal physical activity without treatment, other manifestations of active tuberculosis will develop in more than 90 per cent of the patients. Although many patients in the older age group with primary pleural effusion have tuberculosis, the problem often arises whether the pleural effusion may be a manifestation of pleural involvement by carcinoma. Prompt therapy or palliation is greatly delayed during attempts to obtain a more definitive diagnosis. Difficulties are frequently encountered in the isolation of *Mycobacterium tuberculosis* from the sputum, gastric washings, and pleural fluid of these patients.

At this hospital, only about 25 per cent of presumably tuberculous pleural effusions yield tubercle bacilli. However, many of the patients are initially studied elsewhere, and at the time of transfer to this hospital 50 per cent have only a thickened pleura without free fluid. It has been the policy of the present writers to treat all tuberculin-positive patients with pleural effusion with long-term modified bed rest and combined antimicrobials unless a nontuberculous etiology can be demonstrated. Some mistakes are invariably made by this policy.

MATERIAL AND METHOD

Biopsies of the pleura have been done in 50 consecutive patients presenting with pleural effusion or thickened pleura. Forty-two of these patients were males; 8, females. They ranged in age from two to forty-eight years, and 44 patients were between seventeen and thirty-five years of age. The skin reaction to tuberculin was positive in 41 patients; negative in 8. One patient, a two-year-old male, was not tested. The results in the first 16 of the patients have been published (2).

The first 5 cases were examined by both needle and open biopsy techniques under local anesthesia. The open biopsy was no more difficult and the tissue specimens obtained were so superior that the needle biopsy method was abandoned. The pleura was approached through an intercostal incision, 3 to 4 cm. in length. The parietal pleura was cleaned by blunt dissection, and the visible portion was grasped by an Allis forceps and excised. In most cases, fusion in this area had occurred as early as six weeks from the onset of effusion, but a free pleural space was not considered a contraindication to biopsy even though a

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pneumothorax might result. In the event of a pneumothorax, immediate catheter suction was instituted, and the patient was often not even aware that his lung had collapsed. There were no other complications. In cases of tuberculous involvement of the pleura, the gross appearance of the pleura was often suggestive of the final diagnosis. The patients were ambulated on the day of surgery and there was no change in the appearance of the chest roentgenogram in any of the cases, except the clearing produced by aspiration of free fluid, and a minimal pneumothorax in one case.

In each case, a portion of the fresh specimen was taken for bacteriologic study, which included preparation of direct smears and search for fungi, tissue digestion, concentration and preparation of smears for acid-fast bacilli, as well as culture on Petragnani medium, Sabouraud agar, thioglycolate broth, and blood agar. The remaining tissues were then fixed in formalin, and sections were stained with hematoxylin and eosin. In all patients with granulomatous lesions in the routine sections, further sections were cut and stained with Kinyoun carbol fuchsin and Gomori stains.

For convenience of discussion, the cases are divided into two groups, tuberculous and nontuberculous, on the basis of the final diagnosis.

Results

Group I. Tuberculous Pleurisy

A. Primary pleurisy with effusion (presumably tuberculous): Twenty-three patients met the following clinical criteria: pleural effusion without evidence of nontuberculous etiology or underlying roentgenographically demonstrable pulmonary disease and with a positive tuberculin skin test (table 1).

Fourteen biopsy specimens were histologically compatible with tuberculosis (granulomatous disease with or without caseation), and in 9 of these bacteriologic confirmation was obtained by microscopy or culture of the specimen. It was

TABLE 1

Data on Pleural Biopsy of Patients with Suspected Pleural Tuberculosis

Biopsy	Tuberculin Positive, No Pulmonary Disease		Tuberculin Positive with Pulmonary Disease		Total
Granulomatous histologically	14		4		18
Smear or section positive for acid-fast bacilli		3		1	
Culture positive for acid-fast bacilli		4		0	
Smear and culture positive for acid-fast bacilli		2			
Chronic pleuritis histologically	8		3		11
Smear or section positive for acid-fast bacilli		0		0	
Culture positive for acid-fast bacilli		1		0	
Smear and culture positive for acid-fast bacilli		0		0	
Normal pleura or fibrous tissue	1		2		3
Positive for acid-fast bacilli		0		0	
Total	23	(10)	9	(1)	32

possible to isolate *Mycobacterium tuberculosis* from the pleural fluid in only 2 of these 9 cases. In one additional case, the biopsy specimen showed chronic inflammatory reaction without granulomata, but yielded *Mycobacterium tuberculosis* on culture. Of the 5 patients who yielded histologically compatible but bacteriologically negative specimens, tubercle bacilli were cultured from the pleural fluid of one patient, and 3 patients had positive tuberculin tests only and were negative to coccidioidin and histoplasmin. Although the fifth patient reacted to both tuberculin and coccidioidin, the pleural effusion occurred during a tour of duty in Japan. It was believed, therefore, that the finding of granulomatous changes in the biopsy specimen substantiated the clinical diagnosis of tuberculosis in the 5 patients.

The biopsy specimens from 7 patients with primary pleural effusion showed nonspecific chronic inflammatory reactions but were bacteriologically negative, and one specimen showed normal pleura. The patient with the normal pleura subsequently developed pulmonary disease with cavitation and sputum positive for *M. tuberculosis*, while under antituberculous chemotherapy. Three of these 7 patients reacted strongly to tuberculin, and all other clinical and laboratory studies were negative, including skin tests to histoplasmin and coccidioidin. Some doubt exists as to the tuberculous etiology in the other 4 cases because of either: a weakly positive skin test to tuberculin (second-strength PPD or Old Tuberculin, 1:100); pleural fluid containing a preponderance of polymorphonuclear leukocytes; or changing antistreptolysin titers.[4] The 4 patients are included in the primary (tuberculous) pleurisy group because the suspicion of tuberculosis was sufficient to warrant treatment, and a nontuberculous etiology could not be proved.

B. Pleural effusion with pulmonary tuberculosis: Nine patients had pulmonary disease consistent with tuberculosis, concurrent pleural effusion or blunted costophrenic angle from previous pleural disease, and were tuberculin positive. Biopsy specimens showed granulomatous disease in 4 cases, one specimen of which had acid-fast bacilli on tissue smear. Biopsy in 3 cases showed chronic nongranulomatous pleuritis, and the tissues were negative for *M. tuberculosis*. The diagnosis of tuberculosis in the 7 cases was proved by recovery of *Mycobacterium tuberculosis* in cultures of sputum, gastric washings, and/or pleural fluid. In 2 cases, the biopsy specimens were histologically and bacteriologically normal. The patients gave a history suggestive of an episode of pleural effusion one and a half and two years prior to biopsy. At the time of writing, all bacteriologic studies in these 2 cases are negative.

Case Reports

Case 1 (figure 1). A 48-year-old white male had onset of symptoms of myalgia, fever, chills, night sweats, about July 1, 1955. Symptoms continued on treatment at home, and the patient was hospitalized elsewhere on July 12, 1955. A chest roentgenogram showed pleural effusion on the right, and on July 22, 1955, he was transferred to this hospital.

[4] Since preparation of this manuscript, atypical (chromogenic) acid-fast bacilli were cultured from the pleural tissue of one of these patients.

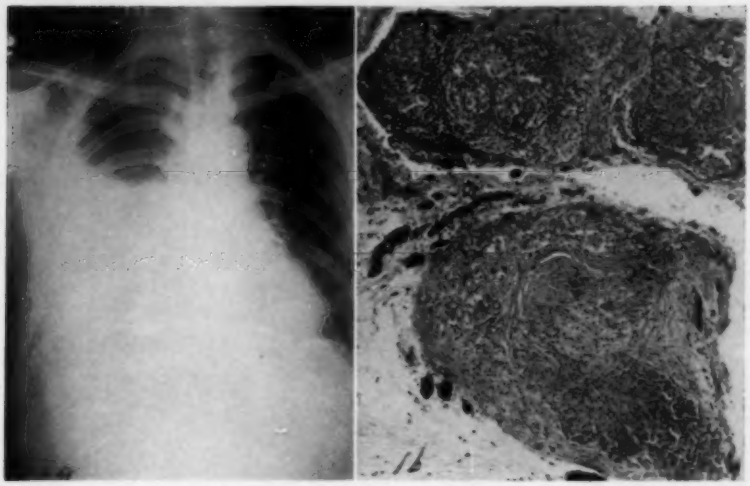

FIG. 1*a*. Pleural effusion in a 48-year-old male. 1*b*. Pleural biopsy four and a half months after onset and two and a half months after start of antituberculous chemotherapy showed granulomatous inflammatory disease, but was negative for *M. tuberculosis*. (Original magnification × 100)

A tuberculin skin test was positive, and coccidioidin and histoplasmin tests were negative. Cytologic and bacteriologic studies of pleural fluid were nondiagnostic. Thoracentesis was difficult because the patient developed a "pleural shock" syndrome when this procedure was attempted. Antituberculous chemotherapy (isoniazid, 300 mg. per day, and streptomycin, 1.0 gm. twice weekly) was started on August 31, 1955. The patient continued to accumulate fluid through September, with gradual resolution subsequent to that. A pleural biopsy was done on November 17, 1955, under local anesthesia. Biopsy tissue showed granulomatous inflammatory disease, but no specific organisms were seen or cultured. The patient was well and the roentgenogram showed only slight blunting of the right costophrenic angle when he was last seen in May, 1957, nine months after completing chemotherapy.

Comment: Neoplastic disease was strongly suspected in this case because of the patient's age and the atypical clinical course. A pleural biopsy was instrumental in dispelling this fear and in establishing an etiologic diagnosis when all other diagnostic methods were not helpful.

Case 2 (figure 2). A 21-year-old female was admitted to another hospital on November 7, 1955, with pleural effusion, following a three-week history of right-sided pleuritic pain, malaise, and low-grade fever. She reacted strongly to second-strength PPD. Pleural fluid and sputum smears were reported negative for acid-fast bacilli, and cultures were negative at the time of transfer to this hospital on November 16, 1955. A brother was said to have had tuberculosis more than ten years previously. The patient had been employed as an attendant in a general hospital from 1949 to 1953.

A chest roentgenogram on admission showed pleural reaction or fluid on the right, extending to the eighth posterior rib. The lung fields were otherwise clear. Fluoroscopy

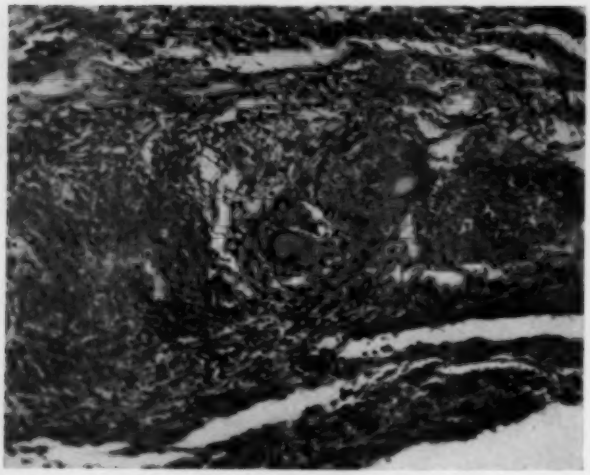

FIG. 2. Pleural effusion in a 21-year-old female. Pleural biopsy was done about two months after the onset of pleurisy and, after ten days of chemotherapy, showed giant cell granulomata. Culture of the biopsy specimen yielded tubercle bacilli. (U. S. Army photograph, original magnification × 200)

showed no evidence of free fluid, and thoracentesis failed to yield fluid. The patient reacted positively to first-strength PPD, coccidioidin 1:100, and histoplasmin 1:100.

Therapy with isoniazid, 300 mg. daily, and aminosalicylic acid, 12.0 gm. daily, was started on December 2, 1955. On December 12 a pleural biopsy was done. Histologic examination of the tissue showed granulomatous disease, consistent with tuberculosis or fungal disease. No organisms were seen on special stains of tissue sections or on smear. However, *Mycobacterium tuberculosis* grew out on culture after nine weeks of incubation. The patient recovered uneventfully and was in good health when last seen in June, 1957, six months after completing chemotherapy.

Comment: This case is typical of many seen at this hospital. Initial studies were done elsewhere and, upon transfer, no fluid was available for study in this laboratory. Although the clinical course was characteristic of tuberculous pleurisy, and treatment would have been prescribed accordingly regardless of failure to obtain bacteriologic proof, the multiple positive skin tests raised the question of fungal disease. The culture positive for *M. tuberculosis* obtained from the tissue left no room for doubt of the diagnosis.

In summary (table 2), pleural biopsy was performed on 32 patients with pleural disease which was considered to be of tuberculous origin. Biopsy tissue was histologically and/or bacteriologically diagnostic in 19 cases (59 per cent).

In the 23 patients without parenchymal disease, the diagnosis of tuberculous pleural effusion was proved in only 4 cases by the use of all methods exclusive of pleural biopsy (17 per cent), and was proved by pleural biopsy in 15 cases (65 per cent). The combination of pleural biopsy with traditional diagnostic methods resulted in a positive diagnosis of tuberculosis in 16 of 23 cases of primary pleural

TABLE 2

PLEURAL BIOPSY IN TUBERCULOUS PLEURAL DISEASE
(Comparison with Traditional Diagnostic Methods)

	Bacteriologic Diagnosis (Without biopsy)	Biopsy Positive for Acid-fast Bacilli	Total Positive Diagnosis
Idiopathic pleural effusion: 23 cases.....	4 (17%)	15 (65%)	16 (70%)
Pleural and parenchymal disease: 9 cases..	7	4	7
Total: 32 cases.....	11 (34%)	19 (59%)	23 (72%)

effusion (70 per cent). In the patients with pulmonary disease, pleural biopsy did not increase the frequency of diagnosis of tuberculosis. However, in 2 cases in this group, the diagnoses were made by pleural biopsy before *Mycobacterium tuberculosis* could be demonstrated by culture of sputum, gastric washings, or pleural fluid; hence, the time necessary for diagnosis was shortened.

The nonspecific inflammatory reaction found in 11 cases was histologically indistinguishable from that found in the pleura of some patients with nontuberculous disease. The finding of nongranulomatous inflammatory tissue cannot be used as evidence against the tuberculous nature of the pleural disease. In one such case, in which a biopsy was done one month after onset of pleural effusion, acid-fast bacilli were recovered by culture of the tissue and, in another case showing this type of reaction, in which a biopsy was done at two weeks, *Mycobacterium tuberculosis* was cultured from the pleural fluid. Normal pleura or fibrous tissue without inflammatory elements was obtained from 2 patients with presumably active pulmonary disease, when biopsies were done one and a half and two years, respectively, after onset of pleural effusion.

Group II. Nontuberculous Pleural Effusion

In 18 cases of pleural effusion of nontuberculous origin, pleural biopsy was diagnostic in one case, helpful in another, and noncontributory in 16 cases.

The biopsy revealed adenocarcinoma in a 27-year-old male believed to have inflammatory disease. In a two-year-old child, it was observed at the time of biopsy that the clinical impression of pleural effusion was erroneous and that the right hemithorax was filled with a cystic mass, demonstrated to be of thymic origin at a later thoracotomy. These two cases have been reported in greater detail elsewhere (2, 3).

The pleural biopsy showed either nonspecific chronic inflammatory tissue or fibrous pleurisy in 14 cases in this group. A patient with known disseminated coccidioidomycosis yielded biopsy tissue histologically indistinguishable from the granulomatous reaction seen in tuberculosis (figure 3). No organisms were identified or cultured. The biopsy tissues from a 24-year-old male with postpneumonic effusion showed chronic perivascular round-cell infiltration. Initial pleural fluid in this case contained 64 per cent eosinophils, but at two months

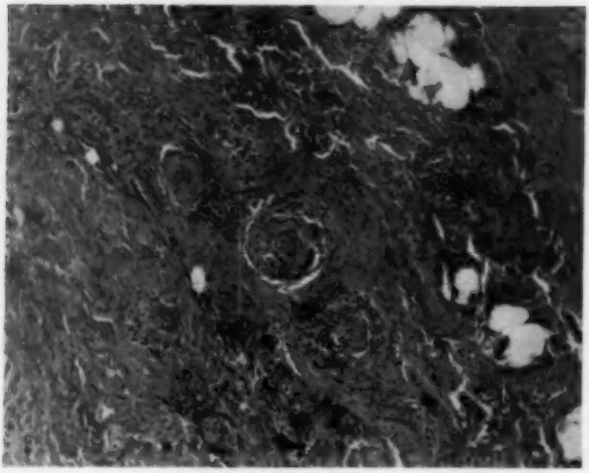

FIG. 3. Giant cell granulomata seen in pleural biopsy specimen of patient with proved disseminated coccidioidomycosis. (U. S. Army photograph, original magnification × 100)

was predominantly lymphocytic. The patient had negative skin reactions to tuberculin, histoplasmin, and coccidioidin. Studies for collagen diseases were negative and he recovered completely without specific therapy.

The final clinical diagnoses in this group of cases are tabulated:

Pneumonia, streptococcal	4
Pneumonia, other	3
Coccidioidomycosis	2
Adenocarcinoma	1
Choristoma of thymus	1
Trauma	1
Undiagnosed	6

DISCUSSION

Biopsy of the parietal pleura was practiced infrequently prior to the report of Sutliff, Hughes, and Rice (4) in 1954. They approached the pleura through a six-inch incision, with removal of a portion of rib, and obtained diagnostic tissue in twenty of twenty-one selected cases, seventeen of which were tuberculous. Small and Landman (5), using a similar technique, found tuberculous tissue in the pleura of five otherwise undiagnosed cases of pleurisy.

Other investigators (6, 7) utilized the Vin-Silverman needle to obtain pleural tissue. Diagnostic tissue was obtained in eleven of twenty-six cases so explored. More recently, Douglas, Carr, and Bernatz (8) reported results of exploratory thoracotomy in twenty-one patients with pleural effusion. Clinical studies had failed to establish a specific diagnosis in thirteen cases (trauma 1, neoplasm 6, tuberculosis 5, rheumatoid pleuritis 1). Stead, Eichenholz, and Stauss (9) reported failure to find evidence of either pulmonary or pleural tuberculosis in

nine of twenty-four cases of presumably tuberculous pleurisy, explored for purposes of decortication.

Thus, several techniques are available for obtaining pathologic information about the diseased pleura. The needle biopsy technique has the advantage of simplicity; it can be performed as an office or ward procedure and does not require the services of a surgeon. On the other hand, it requires the presence of fluid at the time of biopsy, tissue fragments obtained are small, and multiple punctures must be made to obtain specimens for bacteriologic examinations. Open thoracotomy under general anesthesia affords an opportunity to visualize the underlying lung and obtain the best possible biopsy specimens. However, it subjects the patient to added anesthetic risk and morbidity which, in many cases, might not be justified.

The limited open biopsy technique, as described here, and the slightly more extensive procedures, as utilized by Sutliff and associates (4) and by Small and Landman (5), present the disadvantage of requiring the services of a surgeon and the use of the operating room. However, the time required is little more than that required for a needle biopsy, the pleura can be visualized, adequate specimens can be obtained for both histologic and bacteriologic study, morbidity and complications have not been encountered, and the technique is equally applicable regardless of the presence or absence of fluid at the time of biopsy. Fifty per cent of the patients in this series had only residual pleural thickening, often of minimal degree, when first seen at this hospital, and hence would not have been available for biopsy by needle technique.

An attempt to analyze the tuberculous cases with regard to optimal time for biopsy was inconclusive. Diagnostic tissue was obtained as early as one month after onset of pleurisy, and as late as nine months.

Summary

The results of pleural biopsy in 50 cases of pleural disease observed consecutively are presented.

Pleural biopsy by the technique described is a simple and safe procedure. It has proved to be a valuable addition to the traditional diagnostic techniques in pleural disease of tuberculous origin, materially increasing the number of definite diagnoses obtained. However, the finding of nonspecific inflammation or noninflammatory tissue cannot be used to exclude the presence of tuberculosis. The technique may be of value in occasionally establishing a diagnosis of neoplasm or nontuberculous inflammatory disease.

Sumario

La Biopsia Pleural en el Diagnóstico de los Derrames: Resultados en Cincuenta Casos de Afección Pleural Observados Consecutivamente

Preséntanse los resultados de la biopsia pleural en 50 casos de enfermedad pleural observados consecutivamente.

La biopsia pleural con la técnica descrita es un procedimiento sencillo e inocuo. Ha resultado ser una adición valiosa a las técnicas tradicionales de diagnóstico en las afecciones pleurales de génesis tuberculosa, aumentando decididamente el número de diagnósticos bien

definidos obtenidos. No obstante, no cabe usar el hallazgo de inflamación anespecífica o de tejido no inflamatorio para excluir la tuberculosis. La técnica puede resultar útil para establecer de vez en cuando un diagnóstico de cáncer o de enfermedad inflamatoria no tuberculosa.

Resume

Biopsie pleurale dans le diagnostic des épanchements: Résultat dans cinquante cas d'affections pleurales observés en série

Présentation des résultats de la biopsie pleurale dans 50 cas d'affections de la plèvre observés en série.

La biopsie pleurale par la technique décrite est un procédé simple et inoffensif. Sa valeur comme auxiliaire des techniques classiques de diagnostic dans les affections pleurales d'origine tuberculeuse a substantiellement accru le nombre de diagnostics définis obtenus. Néanmoins, la découverte d'un processus inflammatoire non spécifique ou d'un tissu non inflammatoire ne permet pas d'exclure la tuberculose. Cette technique peut être utile pour permettre parfois d'établir un diagnostic de tumeur maligne ou de réaction inflammatoire non tuberculeuse.

Acknowledgment

The writers are indebted to the staff of the Thoracic Surgical Section and of the Histopathology Center, 2349th USAF Hospital, Parks Air Force Base, California, for their assistance in these studies.

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NEEDLE BIOPSY OF THE PARIETAL PLEURA IN TUBERCULOSIS[1]

WILLIAM WEISS

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Introduction

It has long been axiomatic to consider idiopathic pleural effusion in tuberculin-positive subjects to be tuberculous until proved otherwise. Until recently proof of the tuberculous etiology has depended upon the demonstration of tubercle bacilli in the pleural fluid, but this is possible only in a minority of the cases, and confirmation of the diagnosis is usually delayed by the slow growth of the bacilli in culture.

In the last few years diagnosis has been made through biopsy of the parietal pleura by open thoracotomy (1, 2). More recently a simpler approach with the Vim-Silverman needle has been reported (3–5), with equally good results. The needle technique is to be preferred, at least for the initial procedure, because it is simple and innocuous, convenient, repeatable, and lacks the disadvantages of a surgical procedure. The indication for thoracentesis is the indication for needle biopsy.

During the past two years needle biopsy of the parietal pleura in case of effusion has become common practice in the hands of interns and residents at this institution. A report of the results in patients treated as tuberculous in a smaller series of cases has been made (6). The present paper includes the results in a total of 31 cases, 22 previously reported and 9 additional cases.

Method and Material

The method of biopsy is that which has been described by DeFrancis and associates (3). Thoracentesis is done in the usual manner after novocaine infiltration. A 19-gauge needle, attached to a syringe, is inserted while aspirating. When fluid is obtained, the progress of the needle is stopped and a hemostat is clamped on the needle at the skin surface. The needle is then withdrawn and the distance between its point and the hemostat is a measure of the depth of the parietal pleura. This distance, minus 5 to 10 mm., is measured from the point of biopsy needle and marked with another hemostat. The skin is nicked with a scalpel blade and the biopsy needle is inserted to the level of the hemostat. Thus, the point of the biopsy needle will usually lie just outside the parietal pleura. The split needle of the Vim-Silverman assembly may then be inserted to its full depth, the outer needle is pushed in a centimeter or so while the split needle is held, the entire assembly is rotated 360 degrees, and removed. A small cylinder of tissue is usually found within the split needle.

Several pieces of tissue may be obtained by repeating the procedure through the same skin incision and directing the biopsy needle in slightly different directions. In the present series of patients, when the biopsy was unsatisfactory, it was repeated.

The 31 patients included in this series are limited to those who were treated as tuberculous and were a consecutive group of persons admitted to the Department of Pulmonary Diseases with pleural effusions and positive tuberculin tests in whom no etiology for the

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effusion other than tuberculosis was established. They include 12 patients with pulmonary tuberculosis and pleural effusion and 19 with pleural effusion alone. The diagnosis of tuberculosis was confirmed in some patients by pleural fluid cultures (11 cases), sputum cultures (9 cases), open pleural biopsy (one case), or lymph node biopsies (3 cases). In 4 patients, however, the pleural effusion had to be considered idiopathic; these patients were included in the series because they were tuberculin reactors and it was desired to abide by the axiom mentioned in the Introduction.

In this laboratory the usual method of pleural fluid culture consists of direct inoculation of 2 tubes of Löwenstein medium and concentration of 100 ml. of fluid with sodium hydroxide followed by inoculation of a third tube of medium with the sediment. Incubation time is eight weeks, with readings at two-week intervals.

Results

A needle biopsy was considered positive if granulomatous tissue, including caseation and epithelioid cells (usually with Langhans' giant cells), was present. Negative biopsies were required to show evidence of pleural tissue; e.g., inflammatory tissue and/or fibrosis internal to skeletal muscle and/or mesothelial tissue. If this could not be seen, the biopsy was unsatisfactory. When the biopsy revealed only normal tissue, it was often repeated once or twice.

A majority of the patients (23 of 31) yielded positive biopsies, whereas *M. tuberculosis* was isolated from 16 of the 27 patients whose pleural fluid was cultured on one or more occasions. This same ratio, approximately 2:1, held when cases of pulmonary tuberculosis were excluded from the series.

In one patient whose needle biopsy was unrevealing, an open biopsy was done, and this showed tuberculous tissue. The small piece of tissue obtained by needle biopsy was cultured for tubercle bacilli in 2 cases, and one of these was positive. Acid-fast stains of the histologic sections were done a few times and were negative.

A small pneumothorax was noted after biopsy in several patients, but this was rapidly absorbed. No other untoward reactions have been seen.

Discussion

The results in this series indicate that needle biopsy of the parietal pleura is a valuable procedure in the etiologic diagnosis of tuberculous pleural effusion. Any method that will augment the diagnostic yield in "idiopathic" pleural effusion is of increasing importance in view of the fact that tuberculosis is becoming more common in older men. It is in this segment of the population that the differential diagnosis between tuberculous effusion and carcinomatous effusion is a prime consideration. Needle biopsy often provides an early definitive diagnosis.

Needle biopsy of the parietal pleura has also been of occasional value in the diagnosis of malignant effusion, but in such cases cell block of the pleural fluid has usually revealed the diagnosis. Although one procedure may be positive when the other is negative in cases of carcinoma, needle biopsy finds its greatest usefulness in the diagnosis of tuberculous effusions because it is much more often positive than is culture of pleural fluid and because the result is obtained much more quickly.

The specificity of caseating tubercles may be questioned, since these lesions are on rare occasions produced by agents other than tubercle bacilli. However,

nontuberculous causes are sufficiently uncommon so that the error in diagnosis is quite small in patients who are tuberculin reactors.

It must be emphasized that, if the needle biopsy is unrevealing on one occasion, it must not be assumed that either tuberculosis or carcinoma has been excluded. This is particularly true in cases of carcinoma, since the implantation of malignant tumor on the parietal pleura is sometimes not as diffuse a process as seems to occur in tuberculosis of the pleura. A second or third needle biopsy may be more rewarding. If not, then open pleural biopsy may be considered. In the meantime, all diagnostic methods should be brought to bear on the case of effusion because pleural fluid cultures, sputum cultures, or biopsies of other tissues may yield a specific diagnosis when the pleural biopsy is negative.

The frequency with which actual tubercles can be demonstrated in the parietal pleura lends support to the idea that tuberculous pleural effusion is largely due to infection of the pleura rather than to allergic reaction.

Summary

Biopsy of the parietal pleura by the Vim-Silverman needle was done in 31 patients with pleural effusion who were treated as tuberculous. Obvious pulmonary tuberculosis was present in 12 patients and absent in 19. Caseating tubercles were demonstrated in 23 of the 31 patients, whereas pleural fluid cultures were positive for tubercle bacilli in only 16 of the 27 patients whose fluid was cultured. Tuberculosis was established as the etiology in 27 of the 31 patients in the series. Only 4 cases remained "idiopathic" and, as the patients were tuberculin reactors, they received treatment as if the presence of a tuberculous pleural effusion had been demonstrated. Needle biopsy was the single most rewarding diagnostic procedure used in this series of patients. The biopsy is a simple, convenient procedure which appears to be safe and can be repeated.

Sumario

La Biopsia con Aguja de la Pleura Parietal en la Tuberculosis

En 31 enfermos que tenían derrame pleural y a quienes se trataba como tuberculosos se les ejecutó la biopsia de la pleira parietal con la aguja de Vim-Silverman. Había tuberculosis pulmonar manifiesta en 12 de ellos y no la había en 19. Se descubrieron tubérculos en vías de caseación en 23 de los 31 enfermos, en tanto que los cultivos del líquido pleural no fueron positivos para bacilos tuberculosos más que en 16 de los 27 cuyo líquido se cultivó. En 27 de los 31 sujetos de la serie se estableció la tuberculosis como factor etiológico. Sólo 4 casos permanecieron "idiopáticos" y, como los sujetos eran reactores a la tuberculina, recibieron tratamiento como si se hubiera descubierto en ellos un derrame pleural tuberculoso. En este serie de enfermos, la biopsia con aguja fué el procedimiento de diagnóstico más remunerador. La biopsia constituye un procedimiento sencillo y conveniente que parece inocuo y puede repetirse.

Resume

Ponction-biopsie de la plévre pariétale dans la tuberculose

La biopsie de la plévre pariétale par l'aiguille de Vim-Silverman a été effectuée chez 31 malades atteints d'épanchement pleural, qui étaient traités comme tuberculeux. La tuberculose pulmonaire manifeste était présente chez 12 malades et absente chez 19. Des tu-

bercules caséeux ont été observés chez 23 malades sur les 31 étudiés, alors que le liquide pleural n'était positif pour le bacile tuberculeux que sur 16 des 27 malades ou le liquide pleural a été mis en culture. La tuberculose a été établie comme étant le facteur étiologique chez 27 des 31 malades du groupe; 4 cas seulement pouvaient etre considérés "idiopathique" et comme les malades avaient des réactions positives à la tuberculine, ils ont été traités comme si un épanchement pleural tuberculeux avait été démontré. La ponction-biopsie a été la méthode diagnostique qui à elle seule, a été la plus satisfaisante dans ce groupe de malade. La biopsie est un procédé simple commode, qui parait inoffensif et peut être répété.

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PATHERGIC GRANULOMATOSIS (WEGENER'S GRANULOMA) OF THE LUNG[1, 2]

Five Cases of Similar Clinical Courses and Pathologic Findings

R. MAURICE HOOD, HUGH V. O'CONNELL, AND DON L. McCORD

(Received for publication December 11, 1957)

INTRODUCTION

The introduction by Klassen and associates (1) of a technique for biopsy of the lung has made possible a more exact approach to the diagnosis of pulmonary diseases which otherwise do not lend themselves to accurate appraisal by indirect means. Particularly is this true of the diffuse bilateral infiltrative diseases of the lungs which may be indistinguishable from one another by roentgenography. The anatomic study of pulmonary diseases, until recently, has been limited to material obtained at autopsy and from surgical specimens. The types of pulmonary infiltrate under discussion, diffuse bilateral processes, are seldom fatal and have no surgical implications. Therefore, they have not received the clinical or pathologic attention they deserve.

At this clinic, biopsy of the lung has become a routine diagnostic procedure for patients with diffuse pulmonary infiltrations, when other more conventional clinical and laboratory studies have been unrevealing. During the past four years, 5 patients having almost idential clinical and laboratory findings have been found by pulmonary biopsy to have diffuse granulomatous lesions associated with tissue eosinophilia and angiitis. The similarity of the histologic findings in these cases to those of other cases reported in the literature under various names and the surprisingly benign course constitute the basis for this report.

OBSERVATIONS

Case Reports

Case 1. Patient *S. A.*, a 20-year-old man, had a routine 70 mm. photofluorograph of the chest two months prior to admission which revealed an abnormality. At the time of admission the patient complained of minimal shortness of breath after exertion, but was otherwise asymptomatic. A photofluorograph taken two years previously was normal.

The past history revealed no evidence of respiratory disease or exposure to infection.

The physical examination disclosed no abnormalities. The vital signs were normal and the lungs clear. The chest film revealed a diffuse infiltration throughout both lung fields consisting of rounded nodular densities, 1 to 5 mm. in diameter (figure 1). A complete blood count, sedimentation rate, and urinalysis were normal. Numerous sputum smears and cultures for acid-fast bacilli were negative. Skin tests, using PPD, histoplasmin, and coccidioidin, were negative in all dilutions.

A left lung biopsy was performed after examination of biopsy material from a right

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[1] From the United States Naval Hospital, Oakland, California.

[2] The opinions or assertions contained herein are those of the authors and are not to be construed as official or necessarily reflecting the views of the Medical Department of the Navy or the Naval Service at large.

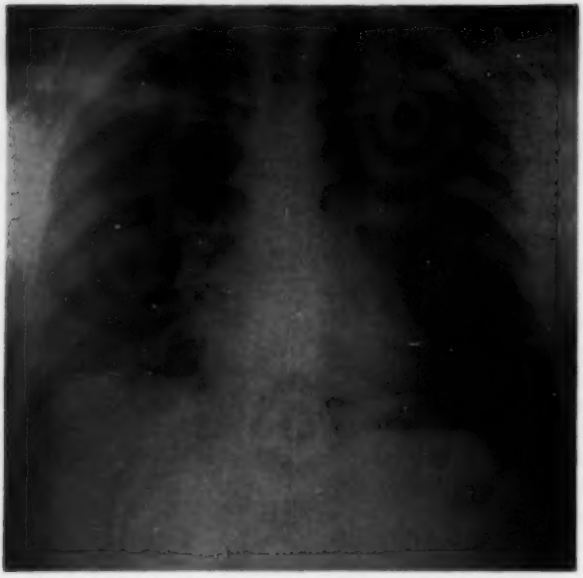

FIG. 1. Chest film of patient of *Case 1* upon admission showed diffuse, rounded bilateral densities measuring 1 to 5 mm.

paratracheal node had been normal. There were scattered small nodules throughout all lobes.

Pathologic Examination

Macroscopic: The specimen consisted of an irregular wedge-shaped piece of lung tissue measuring 2 by 1.5 by 1 cm. Two surfaces were covered by smooth pleura. The sectioned surface showed several small 1 mm. gray flecks which were firmer than the surrounding lung tissue.

Microscopic: Sections showed numerous relatively small granulomatous foci scattered throughout the lung parenchyma (figure 2). These foci were made up of epithelioid cells and myriads of polymorphonuclear leukocytes, of which about one-half were eosinophils. Scattered lymphocytes and plasma cells as well as hemosiderin macrophages were present. The blood vessels showed various amounts of inflammatory involvement. The larger vessels showed much perivascular infiltrate, whereas the smaller ones showed involvement of all layers of their walls. Myriads of hemosiderin macrophages filled the surrounding alveolar spaces. The bronchi and bronchioles were relatively uninvolved (figure 3).

After recovery from the operation the patient was given a twenty-one-day course of cortisone therapy in an oral dosage of 300 mg. daily for three days, then 100 mg. daily for eighteen days. This failed to change the roentgenographic appearance of the lesion. The patient appeared before a physical evaluation board and was retired from the military service.

A recent communication, three years after the initial hospitalization, states that he has remained well and is working full time in a cement plant.

Case 2. Patient *J. B.*, a 22-year-old man, had a routine 70 mm. photofluorograph of the chest, taken two weeks prior to admission, which revealed an abnormality. He had no

FIG. 2. Low-power view of lung biopsy in *Case 1*. Note the focal nature of the lesions. (Hematoxylin and eosin (× 5))

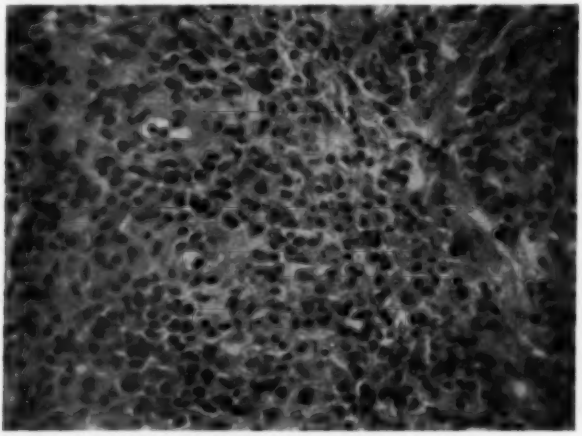

FIG. 3. Lung tissue from patient of *Case 1*, showing granulomatous response with eosinophilia and angiitis. (Hematoxylin and eosin (× 400))

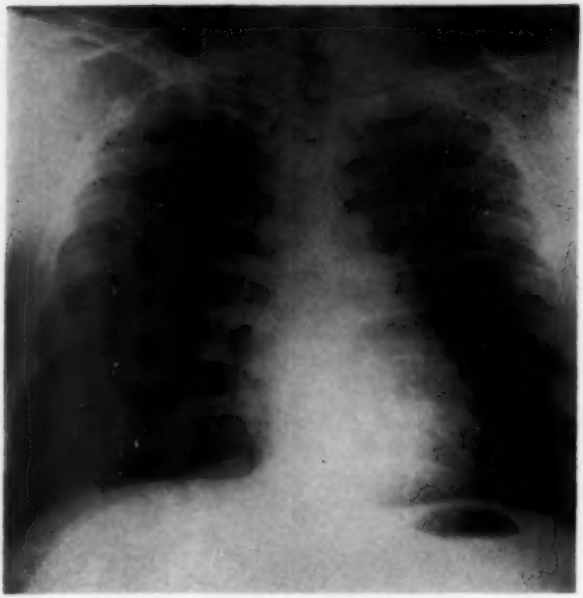

FIG. 4. Chest film of patient of *Case 2* upon admission demonstrated the nodular infiltration throughout both lung fields.

complaints. A roentgenogram of the chest three years previously was normal. The past history revealed an episode of pneumonia during childhood, but there were no sequelae. There was no other past history of respiratory disease or exposure to infection.

The physical examination disclosed no abnormalities. The vital signs were normal and the lungs clear. The chest film showed a nodular infiltration throughout both lungs (figure 4). Several small calcific densities were also present. A complete blood count, urinalysis, total serum protein, albumin-globulin ratio, serum calcium, blood urea nitrogen, serum bilirubin, thymol turbidity, bromsulphalein test, and phenolsulfonphthalein excretion were normal. The skin failed to react to PPD, histoplasmin, and coccidioidin in all dilutions employed. The vital capacity was 2.9 liters, or 62 per cent of normal; and the maximal breathing capacity, 57.6 liters per minute, or 50 per cent of normal.

When no abnormalities were found in biopsy material from a right paratracheal node biopsy, a right lung biopsy was performed. Numerous discrete nodules, 1.5 to 2 cm. in diameter, were found throughout all lobes of the lung.

Pathologic Examination

Macroscopic: The specimen consisted of an irregular portion of moderately firm, light tan to dark reddish-purple tissue having a maximal diameter of 1.5 cm.

Microscopic: Sections showed the small portion of lung tissue partially replaced by a relatively large granulomatous lesion made of epithelioid cells, some lymphocytes, and a few plasma cells. There were many polymorphonuclear leukocytes, most of which were eosinophils. Outside the single larger lesion were a few smaller similar granulomas. The blood vessels within the lesion showed marked occlusive changes with slight polymorphonuclear eosinophilic and round-cell infiltrate within their walls. The surrounding lung parenchyma showed a distorted alveolar pattern (figure 5).

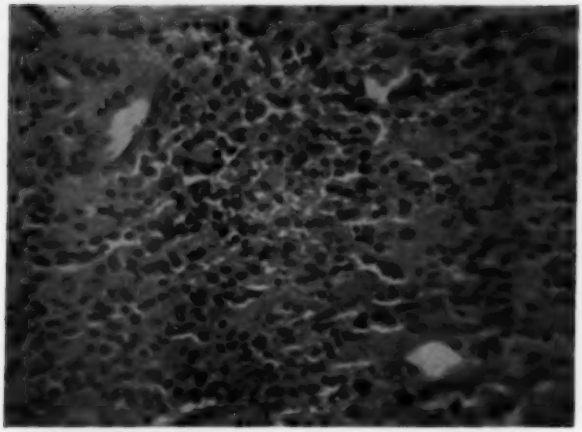

FIG. 5. Lung tissue from patient of *Case 2*, showing resemblance to that in *Case 1*. (Hematoxylin and eosin (× 400))

After recovery from surgery, the patient was given a course of cortisone therapy, consisting of 150 mg. orally for two days and 75 mg. for fourteen days. This had no effect on the roentgenographic appearance of the lesions.

A chest film taken five months after the original one showed no significant change. A recent report of a chest film obtained two years after his discharge from the Navy stated that the previously described infiltrate had completely cleared.

Case 3. Patient *M. O.*, a 35-year-old woman, had complaints of fatigue and exertional dyspnea associated with a nonproductive cough for three months. Because of this and a desire to have a questionable sterility examined, she had an examination which included a roentgenogram of the chest. This showed an abnormality and resulted in hospitalization for further investigation. There were no other complaints referable to the chest.

The past history revealed that at the age of eleven years she had had an undiagnosed episode of arthralgia involving the knees and elbows which was treated with bed rest for six months.

The physical examination discolosed no abnormalities. The vital signs were normal and the lungs clear. The chest film showed an interstitial reticular infiltration involving the lower 80 per cent of both lung fields. No hilar adenopathy was noted (figure 6*A*). The leukocyte count was 13,750 with 38 per cent neutrophils, 60 per cent lymphocytes, and 2 per cent monocytes. The hemoglobin was 12.8 gm. and the hematocrit, 41 volumes per cent. A corrected sedimentation rate was 24 mm. per hour. A repeat leukocyte count and differential were normal one week later. Skin tests, using PPD, histoplasmin, and coccidioidin, were negative in all dilutions. The vital capacity was 2.0 liters, or 68 per cent of normal; the maximal breathing capacity was 75 liters per minute, or 79 per cent of normal.

Right and left paratracheal node biopsies were performed. The pathologic diagnosis was reticuloendothelial hyperplasia. There was no evidence of either specific infection or malignancy. A right lung biopsy was performed. A diffuse nodular infiltration was noted, involving the entire lung, the individual nodules measuring 2 to 10 mm.

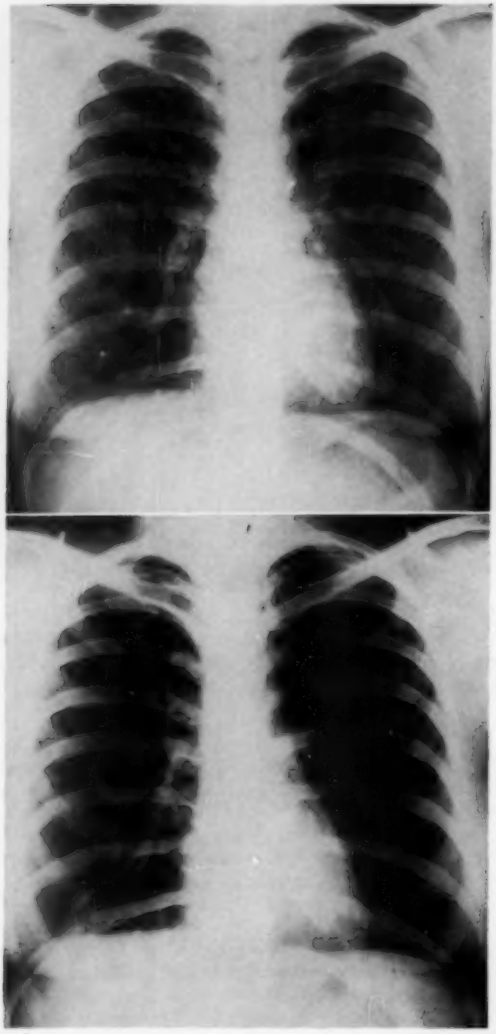

FIG. 6. Reproductions of chest films of patient of *Case 3*. *A* (Upper). Upon admission, an interstitial reticular infiltration involving both lung fields was seen. *B* (Lower). Five months later, this was completely cleared.

Pathologic Examination

Macroscopic: This specimen consisted of a wedge of lung, formalin-fixed, and measuring 2.3 by 1.7 by 1.3 cm. Two surfaces were covered by a smooth, rather dull, grayish-blue, mottled pleura. On section, the pulmonary parenchyma appeared slightly spongelike,

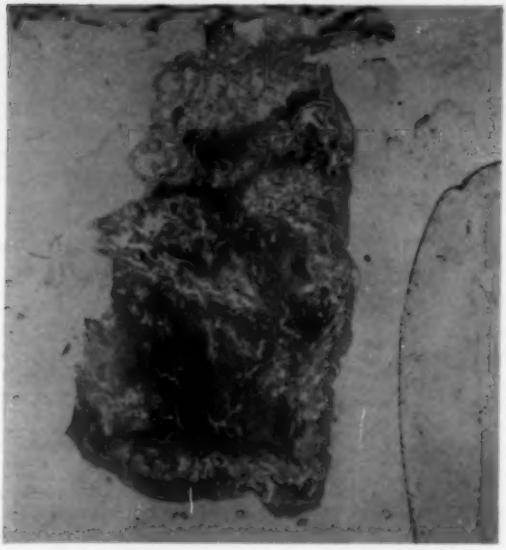

FIG. 7. Low-power view in *Case 3*, showing focal involvement of the lung parenchyma. (Hematoxylin and eosin (× 5))

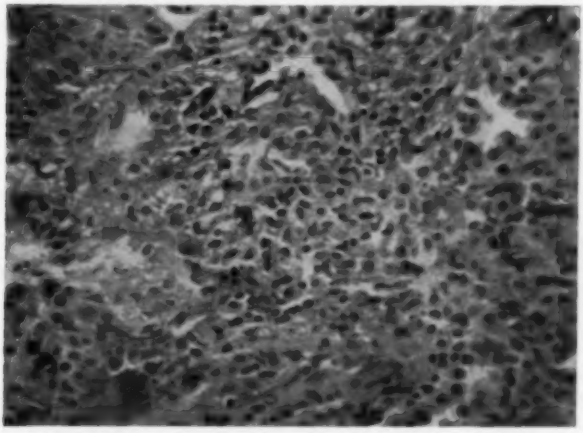

FIG. 8. Lung from patient of *Case 3*, demonstrating similarity to previous cases, notably the eosinophilia and angiitis. (Hematoxylin and eosin (× 400))

tannish-brown, and soft. Contained within this parenchyma were three scattered, firmer gray nodules measuring 0.4 cm. in diameter.

Microscopic: Sections showed marked replacement of lung parenchyma by large and small granulomatous foci (figure 7). The lesions were composed of large masses of epithelioid cells with myriads of inflammatory round cells, mostly lymphocytes and plasma cells with

numerous eosinophils and polymorphonuclear leukocytes. Hemosiderin and lipoid-filled macrophages also were prominent features. The vascular structures showed various changes. Many of the small blood vessels within the granulomatous area showed polymorphonuclear and round-cell infiltrate involving all layers. Others showed marked endothelial proliferation with occlusion of their lumens, and still others showed thickening and sclerotic changes in the muscle layers. The intervening lung tissue showed distortion with some atelectasis in zones immediately surrounding the granulomatous foci. The alveolar spaces contained numerous debris-laden macrophages. The bronchi and bronchioles contained numerous macrophages also (figure 8).

Five months after admission a chest film showed complete resolution of the previously described interstitial infiltration (*see* figure 6*B*).

Case 4. Patient *S. B.*, a 29-year-old woman, had a chest film reported as abnormal during the course of a sterility study. She had noted increasing fatigue and a 21-pound weight loss during the preceding year. The only complaint referable to the chest was a dry hacking cough thought by the patient to be due to smoking 30 cigarettes daily. During the three weeks prior to admission she had a fever up to 101°F. A chest film obtained three years before the onset was normal.

Past history revealed the presence of a diffuse nontoxic enlargement of the thyroid gland for several years.

The physical examination disclosed normal vital signs. There was a moderate, diffuse enlargement of the thyroid gland. A 1-cm. nodule was noted in the right side of the breast. The lungs were clear. A chest film demonstrated a generalized reticular infiltration in both lung fields. No hilar adenopathy was noted (figure 9*A*). The complete blood count and urinalysis were normal. On January 26, 1956, the vital capacity was 2.56 liters, or 88 per cent of normal; the maximal breathing capacity was 145.5 liters per minute. On February 28 the vital capacity was 1.98 liters, or 62 per cent of normal; the maximal breathing capacity, 118 liters per minute.

After a negative right paratracheal node biopsy, a right lung biopsy was performed. The lung was filled with innumerable nodules from 2 mm. to 1 cm. in diameter, and a biopsy was taken from the superior segment.

Pathologic Examination

Macroscopic: The specimen consisted of a wedgelike segment of lung, formalin-fixed, and measuring 2.5 by 1.3 by 1 cm. Two surfaces were covered by dull gray, slightly wrinkled, thin pleura. The sectioned surface was spongelike and mottled, showing various shades of gray and tannish-brown. The specimen appeared firm and homogeneous.

Microscopic: Sections showed scattered granulomatous foci replacing lung parenchyma (figure 10). These foci varied considerably, the smaller ones being the size of 3 to 4 alveolar spaces and the larger ones occupying several low-power microscopic fields. The lesions were composed of masses of epithelioid cells, numerous lymphocytes, and macrophages. Many polymorphonuclear leukocytes, the majority of which were eosinophils, and a few plasma cells were also seen. Multinucleated giant cells of the Langhans' type were absent. The bronchial and alveolar vessels showed various changes; many were uninvolved while some showed chronic inflammatory infiltrate involving all layers, this being more noticeable within the granulomatous foci. Others showed some thickening with myxomatous and basophilic degenerative changes and without observable inflammatory infiltrate. The intervening lung parenchyma showed changes varying from atelectasis to areas of compensatory emphysema. The small bronchi and bronchioles contained numerous hemosiderin and debris-laden macrophages (figure 11).

Following recovery from the operation, the patient was discharged to resume her usual activities as an office receptionist. Follow-up chest films revealed a

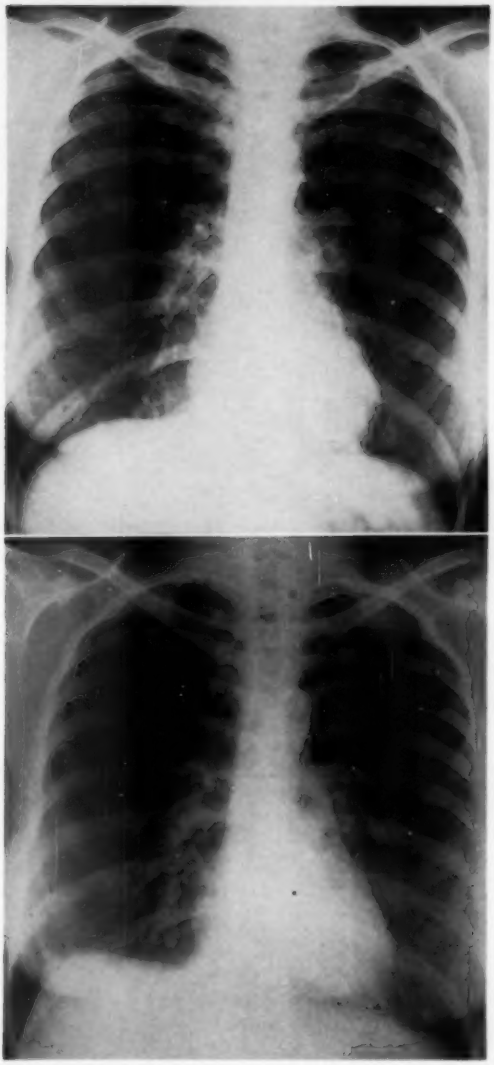

FIG. 9. Reproductions of chest films of patient of *Case 4*. *A* (Upper). Upon admission, a generalized reticular infiltration diffusely distributed throughout both lungs was seen. *B* (Lower). Sixteen months later, the lesions were completely cleared.

FIG. 10. Low-power view of lung tissue from patient of *Case 4*, again showing the characteristic focal nature of the lesions. (Hematoxylin and eosin (× 5))

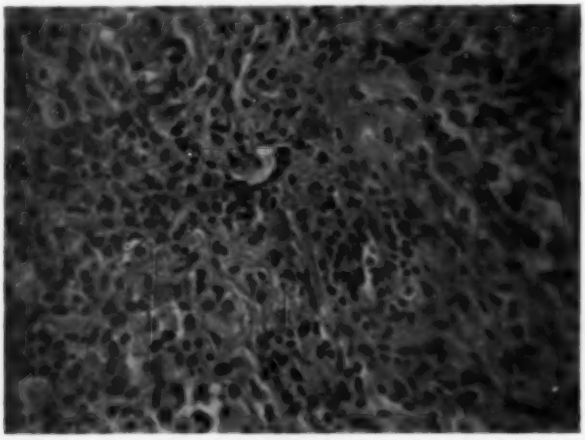

FIG. 11. Lung tissue from patient of *Case 4*, showing under higher magnification the marked eosinophilia and angiitis. (Hematoxylin and eosin (× 400))

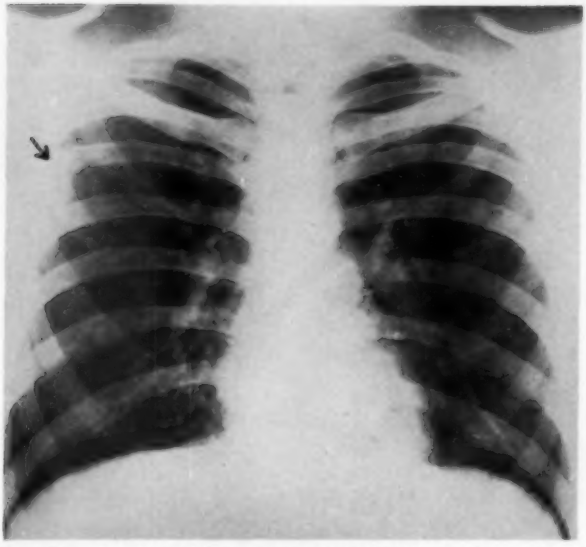

FIG. 12. Chest film of patient of *Case 5* upon admission, illustrating a pathologic fracture of the right fifth rib and a patchy, nodular infiltration distributed throughout both lung fields.

gradual clearing of the pulmonary infiltrate to the extent that it had completely disappeared ten months after the original film showing the disease, and continued to be clear for the following six months (*see* figure 9*B*). The patient has remained asymptomatic since shortly before admission.

Case 5. Patient *R. H.*, a 19-year-old man, was admitted to the hospital because of a severe pain beneath the tip of the right scapula which was aggravated by deep breathing or coughing. He had no other symptoms. There was no history of trauma, weight loss, pulmonary disease, or malaise.

Physical examination revealed tenderness over the region of the right fifth rib posteriorly, produced by direct pressure or anteroposterior compression of the rib cage. The lung fields were clear except for slightly distant breath sounds. The chest film showed a patchy nodular infiltration throughout both lung fields. In the fifth rib at the posterior axillary line was a 2 by 3 cm. osteolytic lesion through which there was a pathologic fracture (figure 12). The complete blood cell count and urinalysis were normal. The sedimentation rate was 15 mm. per hour. The uric acid, serum calcium, serum phosphorus, and alkaline phosphatase were normal. The total proteins were 6.7 gm. per 100 ml., with 3.4 gm. per 100 ml. albumin and 3.3 gm. per 100 ml. globulin. The test for Bence Jones protein was negative, as was a skin test for PPD. A bone marrow examination was reported normal.

After a negative right paratracheal node biopsy, an excision biopsy of the involved segment of the right fifth rib and a biopsy of the right lung were performed. Throughout the lung were innumerable nodules, 2 to 3 mm. in diameter. After recovery from the operation the patient was given thirty days' convalescent leave. Films taken after he returned from leave demonstrated no clearing of the nodular densities in the lung fields.

FIG. 13. Low-power view of lung biopsy from patient of *Case 5*, demonstrating the scattered focal nature of the granulomatous lesions. (Hematoxylin and eosin (× 5))

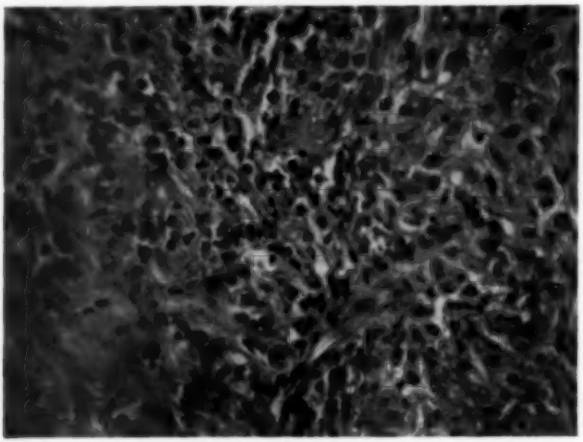

FIG. 14. *Case 5*, showing the histologic similarity to the other cases. (Hematoxylin and eosin (× 400))

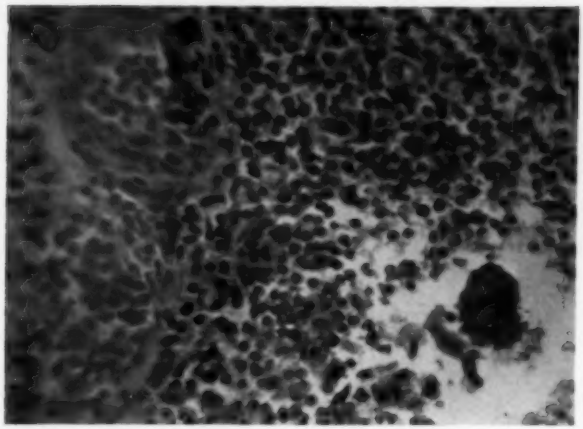

FIG. 15. *Case 5*, demonstrating the eosinophilic granulomatous nature of the rib lesion. Hematoxylin and eosin (× 400))

Pathologic Examination

Macroscopic: The specimen consisted of 2 portions. The first was a pyramidal segment of lung tissue measuring 2 by 1 cm., and one surface was covered with a glistening membrane which varied in color from tan to dark brown. The other surfaces were granular in appearance and varied in color from areas of light tan to dark brown.

The second specimen consisted of a segment of rib measuring 10 cm. in length and averaging 2 cm. in diameter. Attached to the periosteum were multiple fragments of muscle and connective tissue. At the central portion the rib was expanded to form a fusiform swelling measuring 3 by 2 cm. The periosteum overlying this fusiform area was somewhat looser than that over the adjacent bone, and softening and fracture of the bone were noted at this point. Upon sectioning longitudinally, the marrow was dark red while the central portion, corresponding to the fusiform swelling, was brownish-tan, homogeneous, and much firmer in consistency.

Microscopic: Sections of the first specimen showed lung tissue containing small scattered granulomatous foci consisting of masses of epithelial cells, fibroblasts, and macrophages, together with myriads of eosinophils. Within the granulomatous areas and at their margins, the blood vessel walls contained some polymorphonuclear infiltrate. The surrounding lung tissue was compressed (figures 13 and 14).

Sections of the bone showed osteolysis by a granulomatous process, the outstanding feature being numerous eosinophils. Macrophages, fibroblasts, and epithelioid cells were also prominent. The blood vessels showed various amounts of angiitic involvement. The granulomatous process could be seen extending into the surrounding soft tissue, including the skeletal muscle (figure 15).

DISCUSSION

Reports on 5 patients have been presented, three men and two women, less than thirty-five years of age. All have been found by roentgenographic examination to have diffuse, fine, nodular, pulmonary infiltrations. Three have been generally asymptomatic during the entire period of observation. One patient (*Case 3*) had complaints of malaise, fever, and weight loss for four months prior

to hospitalization. This patient became asymptomatic while under observation and without any specific therapy.

Four of these patients have shown no clinical or laboratory evidence of involvement by a granulomatous process of the upper respiratory passages, kidneys, the vascular system outside the lung, or of any other organ system. The remaining patient (*Case 5*) was the only exception, having in addition a solitary rib lesion histologically typical of eosinophilic granuloma. None of these patients has had symptoms, physical signs, or laboratory findings of allergic disease states, nor did any of them show peripheral eosinophilia.

Respiratory ventilation studies in *Cases 3*, *4*, and *5* have failed to show any marked ventilatory defect. This was presumed to have been due to the relatively small amount of total lung parenchyma involved by the granulomatous lesions.

Roentgenographic examinations have shown a diffuse, bilateral, finely nodular infiltrate which cleared completely in *Cases 2*, *3*, and *4*. No residual shadows or fibrosis have persisted. In *Case 1*, follow-up films were not available. There has been no hilar or mediastinal lymphadenopathy in any patient. *Case 5* is a patient currently hospitalized.

Special diagnostic studies—including multiple examinations of the sputum and gastric contents for fungi and acid-fast bacilli; skin-testing for coccidioidomycosis, histoplasmosis, and tuberculosis; and paratracheal lymph node biopsy and culture—have all been negative. A lung biopsy was performed in each case.

The 5 patients of the present report shared several things in common. All showed granulomatous foci, the striking features being eosinophilia and angiitis. *Case 1* was originally considered to be an example of "organizing pneumonitis with eosinophilia." *Cases 2*, *3*, and *4* were originally thought by one of the writers (HVO'C) to be compatible with "Wegener's granulomatosis" although some viewers considered *Case 3* to represent eosinophilic granuloma of the lung. The most recent, *Case 5*, showed a pulmonary lesion identical in all respects with the first 4 cases. In addition, the bone lesion was identical histologically with the pulmonary lesion and, if examined separately, undoubtedly would be considered by many pathologists to be an example of eosinophilic granuloma of the bone. The pulmonary lesions in all 5 patients have certain characteristics which fit them into the pattern variously described under a host of syndromes, all previously described in the literature and considered in the discussion.

In spite of the angiitis present in all of these patients, necrosis was not a prominent feature. The inflammatory reaction within the vessel walls was characterized by polymorphonuclear and round-cell infiltration in all layers. Endothelial proliferation was present in some but not all. The bulk of the granulomatous masses was composed of aggregates of epithelioid cells, macrophages, lymphocytes, plasma cells, and myriads of eosinophils. The surrounding lung parenchyma not involved in the granulomatous process showed some alveolar distortion and compensatory emphysema.

A review of the literature reveals that Klinger, in 1931, first described a granulomatous process involving the lung and kidneys with vascular lesions similar to those of periarteritis nodosa (2). Wegener reported similar cases and

recognized that these were different from those usually referred to as periarteritis (3, 4). His name has since been associated with a syndrome consisting of necrotizing granulomatous lesions involving the upper and lower respiratory tracts and the kidney. There have been about forty cases recorded, and a recent review by Fahey, Leonard, Churg, and Godman indicated that all of these have terminated fatally within a few months (5).

Histologically, the cases reported here are strikingly similar to those reported as Wegener's granulomatosis despite the fact that the clinical course has been in no way similar.

That confusion exists in the minds of clinicians and pathologists alike is evidenced by numerous other reports which, when critically compared, are either identical histologically or are so alike as to suggest at least a causal relationship. Various authors have attached some of the following names to diseases which in retrospective comparison are probably closely related: Periarteritis nodosa of the lung (2), rhinoserous granuloma (3, 4), Wegener's granuloma (5), eosinophilic granuloma, lupus erythematosus, rheumatic pneumonitis, Löffler's syndrome (6), necrotizing granulomatosis and angiitis of the lung (7, 8), granulomatous glomerulonephritis, and allergic granuloma of the lung (9).

Despite histologic and in some cases roentgenographic similarity, the clinical course of the patients varies widely. The cases reported as Wegener's granulomatosis, with a single exception, have been from diagnoses made at autopsy; at the opposite extreme are those referred to as Löffler's syndrome with only transient lesions. Still other cases, such as those diagnosed as periarteritis nodosa, have different clinical courses.

Pathologically these various lesions do not differ sufficiently, except in extent and degree, to be viewed as separate diseases. It is therefore seemingly unwise to insist that various clinical findings and courses require that they be considered as unrelated diseases of different etiology. Perhaps with more study and experience these conditions will ultimately prove to have separate etiologies. Should this be established, there will be adequate reason to classify and name the syndromes on a reasonable basis.

That these conditions or syndromes are basically diseases of tissue hypersensitivity is suggested by the papers of Fahey and associates (5), Fienberg (7, 8, 10), and of Churg and Strauss (11). Many writers agree with this concept. The finding of eosinophils in histologic section is usually considered as a response to some antigenic substance on an allergic or hyperergic basis. Assuming the etiologic theory to be true, the writers have been impressed with the approach of Fienberg to the classification and nomenclature of these diseases (10). Because of the obvious impossibility of differentiating between closely related diseases of unknown etiology, he has suggested that the term "pathergic granulomatosis" be utilized. The word "pathergy" is defined as "the totality of morbid phenomena which can be produced by a state of altered tissue reactivity."

Fienberg further suggests the division of the diseases into two groups, depending upon the extent and location of the lesions. The first group he identifies as generalized and includes those having widespread lesions or multiple organ

involvement. In the second group, he places those in which the lesions are confined to a single organ or organ system. The patients in the present report fall into his second group, with the exception of the patient of *Case 5*, who, in addition to the pulmonary involvement, had a solitary rib lesion.

Summary

The cases of 5 patients found to have similar clinical, roentgenographic, and histologic characteristics have been presented. The pathologic examination of pulmonary tissue removed by lung biopsy from these patients has consistently shown necrotizing granuloma with marked tissue eosinophilia and angiitis. These findings have been reported in the literature under various names and by numerous writers. The patients of this report, although having histologic characteristics of Wegener's granuloma, have had benign clinical courses. The classification and nomenclature of Fienberg have been discussed and are recommended for general use until the etiology of this group of diseases is clarified. The diagnostic value of lung biopsy has again been demonstrated.

Sumario

Granulomatosis Patérgica (Granuloma de Wegener) del Pulmón

Preséntanse los casos de 5 enfermos que manifestaban semejantes características clínicas, radiográficas e histológicas. Se describen el examen patológico y los hallazgos histológicos. El examen patológico del tejido pulmonar extirpado por la biopsia a estos enfermos ha revelado constantemente granuloma necrotizante con acentuada eosinofilia y angiítis en los tejidos. Estos hallazgos han sido expuestos en la literatura con varios nombres y por numerosos autores. Los enfermos, descritos ahora, aunque mostrando las características del granuloma de Wegener, han tenido una evolución clínica benigna. Se discuten la clasificación y la nomenclatura de Fienberg, recomendándose para empleo general hasta que se esclarezca la etiología de este grupo de dolencias. Queda una vez más demostrado el valor diagnóstico de la biopsia pulmonar.

Resume

Granulomatose pathergique (granulome de Wegener) du poumon

Les observations concernant 5 malades présentant des caractéristiques cliniques histologiques et radiologiques similaires sont présentées. Les caractéristiques de l'examen anatomo-pathologiques et histologiques sont décrites. L'examen anatomo-histo-pathologique du tissue pulmonaire reséqué par biopsie chez ces malades a constamment révélé le granulome nécrosé avec éosinophilie et angéite marquées des tissus. Ces manifestations ont été rapportées dans la littérature sous de nombreuses dénominations et par de nombreux auteurs. Bien que les signes histologiques du granulome de Wegener aient été présents chez les malades concernés dans ce rapport, le cours clinique de la maladie a été bénin. La classification et la nomenclature de Fienberg sont commentées et il est conseillé d'en faire usage d'une façon générale jusqu'au moment où l'étiolgie de ce groupe d'affections soit élucidée. La valeur diagnostique de la biopsie pulmonaire a été de nouveau démontrée.

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INTRACRANIAL CALCIFICATION FOLLOWING TUBERCULOUS MENINGITIS IN CHILDREN[1]

JOHN LORBER

(Received for publication December 26, 1957)

Introduction

Calcification of intracranial tuberculous lesions was very rare before the introduction of effective antituberculous drugs. In a detailed review of the literature of the past one hundred years, only forty-nine instances of proved or suspected intracranial calcification in cerebral tuberculemas were found (1).

After the introduction of streptomycin, intracranial calcification following tuberculous meningitis became frequent. Calcification was first noted in patients surviving with neurologic sequelae (2, 3). The first systematic study of roentgenograms of the skull of children who recovered from tuberculous meningitis was published in 1952 (1). At that time the present writer reported that intracranial calcification was detectable in seventeen of twenty-five children who survived and had been followed for two and one-half to four and one-half years from the onset of the disease. In addition, calcification was detected at necropsy in two children who died twenty-one months and twenty-nine months, respectively, from the beginning of treatment.

Following these papers, several other series of cases have been published (4–13) (table 1) as well as individual case reports (14–17) which confirm the high incidence of intracranial calcification, particularly in patients treated in the advanced stage of the disease and in those who have been observed for several years. Numerous cases have been described in which neurologic or endocrine sequelae were associated with intracranial calcification, but there is no evidence that such calcification is more common in children with sequelae or that the calcification is directly responsible for them.

The objects of the present investigation were as follows: to extend the knowledge of intracranial calcification following tuberculous meningitis in children by the study of a much larger series of cases; to attempt to determine whether the addition of the newer antituberculous drugs, isoniazid, PAS, and other adjuvants, such as intrathecal tuberculin, influenced the incidence of such calcification; to correlate the incidence of neurologic and mental sequelae with intracranial calcification; and to attempt to determine the prognostic significance, if any, in relation to late relapses of meningitis.

Material and Methods

One hundred and thirty children were studied who developed tuberculous meningitis between September, 1947, and June 30, 1955, and in whom roentgenograms of the skull

[1] From the University of Sheffield's Children's Hospital, Western Bank, Sheffield 10, England.

TABLE 1

THE INCIDENCE OF INTRACRANIAL CALCIFICATION FOLLOWING TUBERCULOUS MENINGITIS IN CHILDREN

Author	Year	Number of Cases	Length of Follow-up	Number with Calcifications	Per Cent	First Appearance	Basal	Central
			years					
Lorber (1)	1952	27	2½–4½	19	70	19/12	12*	10*
Russell and MacArthur (4)	1953	14	4–5½	10	70	23/12	8	2
Garsche (5)	1953	35	Approximately 2–5	12	34	12/12	12	
Plettenberg (6)	1953	31	Approximately ½–4	6	19	19/12	6	—
Seringue and J. Marie (7)	1953	23	Approximately 3–5	2	9	?	?	?
Denys *et al.* (8)	1953	122	Approximately 2–5	20	16	?	12+	Some
Lefebvre *et al.* (9)	1954	68	2–6	16	24	?	Most	Some
Latorre *et al.* (10)	1954	31	2–6	11	36	?	Most	Some
Maggioni (11)	1955	50	1 year, 1 month–6	10	20	2 years, 4 months	Most	
Caffaratti and Lanza (12)	1956	26	2–7	6	23	2½ years	6	—
Aleksandrova (13)	1956	80	1–8	21	26	?	Most	

* Including 3 patients with both types of calcification.

were obtained on admission and at least once twenty-one months or more after the onset of their meningitis. These 130 children were members of a consecutive series of 131 children who had been treated in the Children's Hospital, Sheffield, and who survived for a minimum of twenty-one months. The only exception was a girl who became a decerebrate idiot, and in whom it was not possible to obtain a postmeningitic roentgenogram of the skull. This series of cases, therefore, can be considered to be fully representative, without selection or bias. Because of the completeness and the large size of the series, as well as the length of observation, it is considered that the results to be reported here are likely to be a true reflection of the incidence and nature of intracranial calcification following tuberculous meningitis in children who were treated by the methods to be described.

Physiologic calcification in the pineal gland, the choroid plexuses, and in the falx cerebri has not been considered.

Of the 130 children, 120 are alive and 119 of them are free from evidence of active intracranial infection. One suffered a recent relapse of the meningitis and is again under treatment. All have been followed for a minimum of two years. Sixteen of the survivors have neurologic sequelae which will be discussed in detail later.

Ten children died after a prolonged or recurrent illness from twenty-one months to six years after the beginning of their treatment. Of the 10 children who died, 5 had gross neurologic lesions before their final fatal illness, consisting, in one case, of partial blindness with optic atrophy and in 4 of extreme hydrocephalus with amentia. In 5 cases, death was due to

continued tuberculous meningitis or to late relapse. Four died of the complications of their severe hydrocephalus, and death was therefore indirectly attributable to their meningitis. One died of meningococcal septicemia seven years after her recovery from the tuberculous meningitis.

As this investigation started in 1950, children who were treated in 1947–1949 did not have routine postmeningitic roentgenograms until almost three and one-half years had elapsed after the onset of their disease. After 1950, however, routine roentgenograms of the skull were taken in nearly all cases in the anteroposterior and lateral positions (using other views when necessary), at the latest eighteen months after the onset of the disease and at approximately six- to twelve-month intervals subsequently.

All of the 130 children received intrathecal streptomycin treatment for various periods. The duration of intrathecal treatment decreased when first PAS and later isoniazid were added to the treatment. All patients received intramuscular streptomycin for a period of six months or more. Forty-two patients (in the early years) were treated with streptomycin as the only antimicrobial, 40 received PAS in addition, and the last 48 received isoniazid, streptomycin, and PAS. Fifteen children were given intrathecal tuberculin, and 6, intrathecal streptokinase as adjuvants. Full details of the treatment of all of the patients have been described in previous publications (18–22).

On admission, 33 children (31 survivors) were considered to be in the early stage of the disease, 79 (75 survivors) in the intermediate stage, and 18 (14 survivors) in the advanced stage. The small number in the advanced stage reflects the poor survival rate in this group.

Results

General

Until November, 1957, pathologic intracranial calcification was discovered during life in roentgenograms of the skulls of 63 children (48.4 per cent). In those who had serial roentgenograms, it was possible to observe the gradual development of the calcification, early traces of which were frequently detected only in retrospect after more definite evidence had been obtained. In one child, the earliest detectable calcification occurred fifteen months after the onset of the meningitis. The detailed timetable of the first appearance of calcification is shown in table 2, which also shows the time of the last roentgenogram in relation to the onset of the disease of the 67 patients who showed no evidence of intra-

TABLE 2

The Timetable of the Roentgenographic Appearance of Intracranial Calcification in 130 Patients

Months After Admission	Patients with Calcification		Patients Without Calcification at Time of last Roentgenogram	
	Number	Per Cent	Number	Per Cent
15–24	16	12.4	2*	
25–36	33	25.5	18†	
37 or more	14	10.8	47	
Total	63‡	48.4	67	51.6

*, † Includes 1 and 3 children, respectively, who died during these periods.

‡ Seven patients had not been examined roentgenographically for 24 months and 5 others for 36 months.

cranial calcification. It may be seen that calcification became demonstrable within thirty-six months of the onset in 49 children, or more than four-fifths of the patients with bacteriologically proved tuberculous meningitis. The true proportion is undoubtedly higher because 5 patients were not examined roentgenographically before the thirty-seventh month. Among the 67 patients with no calcification, there were 47 whose last roentgenograms were taken between the thirty-seventh month and seven and one-half years. There were 11 patients who had normal roentgenograms at thirty-six months. Four patients died less than three years from admission, and only 5 living children with normal skull roentgenograms have been followed roentgenographically for less than three years.

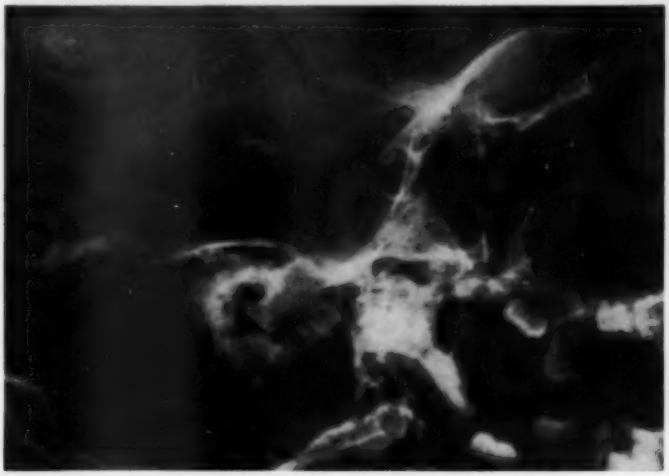

FIG. 1.

FIG. 1A.

FIG. 1, 1A. Solitary calcified plaque above the posterior clinoid process. No sequelae.

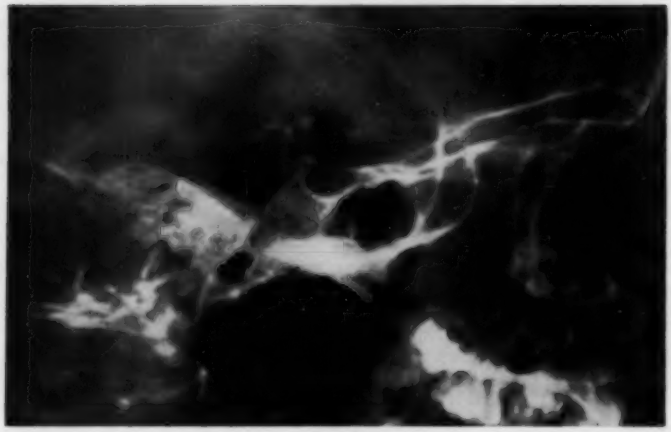

FIG. 2.

FIG. 2A.

FIG. 2, 2A. Irregular, broken-up plaque above the posterior clinoid process with a single lesion over the anterior clinoid process. No sequelae.

As calcification very rarely occurred after this time in the group of patients with proved tuberculous meningitis, it seems unlikely that there would be a substantial increase in the proportion of children with calcification. Disappearance of calcification has not occurred in any case.

Sites of Calcification

Two main sites of pathologic calcification were found. The more common site was the basal meninges. Less commonly the calcification was intracerebral. In 3 children calcification occurred in both places.

Meningeal calcification (roentgenographic description): Calcification occurred most commonly in the meningeal exudate, which is usually seen at the base of the brain in tuberculous meningitis. Such meningeal calcification was observed in 49 cases. In most cases this was in the vicinity of the sella turcica, usually above the anterior or posterior clinoids or extending further back. It consisted of a single lesion in 9 cases, but in the large majority several smaller lesions formed clusters of calcification either as a solitary plaque (figure 1, 1A), or more commonly as an irregular, broken, branching plaque (figure 2, 2A). Sometimes the

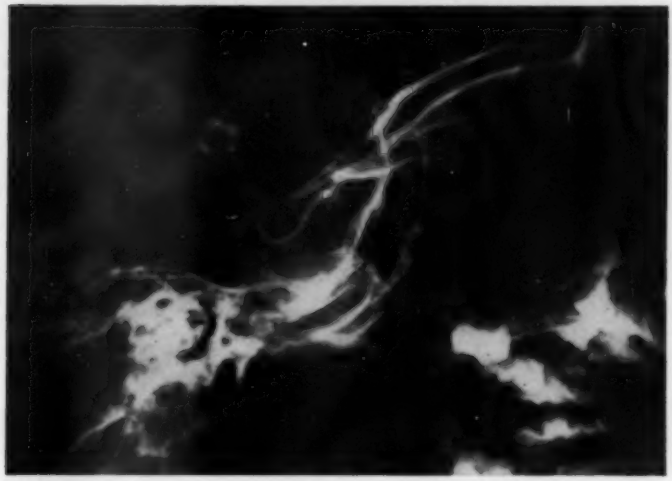

FIG. 3.

FIG. 3A.

FIG. 3, 3A. Clusters of calcification forming a ring above the posterior clinoid process. Child recovered from hemiplegia. Relapse of the meningitis two years later.

plaques formed a ringlike shadow (figure 3, 3A) or a small conglomerate mass just above the posterior clinoid. Their situation closely corresponded to that of the cisterna chiasmatis, in which the cistern was outlined by air. It is difficult to demonstrate calcification in anteroposterior views of the skull in moderate cases but, when it is visible, it is found on either one or both sides, very near to the midline. In more extensive cases it can be clearly shown in anteroposterior and lateral views that the calcification extends around the midbrain and occupies the cisterna chiasmatis (figures 4, 4A; 5, 5A; 6, 6A; and 7, 7A). This may be

FIG. 4.

FIG. 4A.

FIGS. 4, 4A; 5, 5A. Lateral and anteroposterior views. Massive intracerebral calcification and meningeal calcification occupying the basal cisterns and the cisterna ambiens. Normal child.

particularly well shown on Townes[2] views (figures 8, 8A). These situations are of course well known to be the commonest sites for the obstruction of the flow of the cerebrospinal fluid during treatment of the active phase of the meningitis, as may be well demonstrated by pneumoencephalograms (23).

Much less commonly the exudate calcifies in the meningeal spaces of the Sylvian fissure. Such calcification was seen in only 3 cases (figures 6, 6A; 9, 9A).

[2] A 30 degree fronto-occipital view.

FIG. 5.

FIG. 5A.

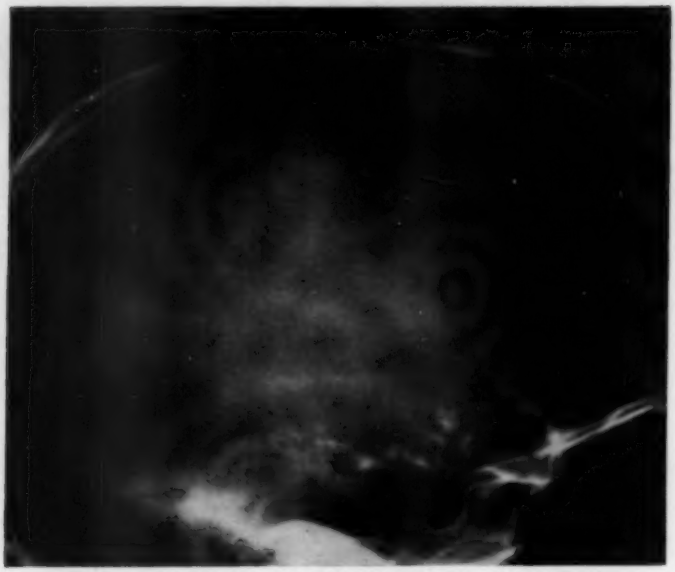

FIG. 6.

FIG. 6A.

FIG. 6, 6A. Extensive meningeal calcification extending over the whole of the sella and behind it, as well as calcification in the Sylvian fissure. Blind child. (*Case 16*, table 6).

The Incidence of Meningeal Calcification in Relation to the Stage of the Disease on Admission and to Treatment

Thirty-three of the 130 patients were in the early stage of the disease on admission. Seven (21 per cent) of these developed meningeal calcification as compared with 32 (40 per cent) of 79 with intermediate disease and 10 (55 per cent) of 18 with advanced disease (table 3). Thus, there was a progressively higher incidence of calcification with increasing severity of the disease on admission. More efficient treatment, especially after the introduction of isoniazid,

apparently resulted in an appreciable reduction of the over-all incidence of intracranial calcification (table 4). Of 42 patients treated with streptomycin alone, 20 (48 per cent) developed meningeal calcification, as compared with 37 per cent of the 40 treated with streptomycin-PAS, and 29 per cent of the 48 treated with isoniazid, streptomycin, and PAS.

At first sight these values might be taken to indicate that progressively more efficient treatment led to a more complete resolution of the meningeal exudate, so that less necrotic residue was left for future calcification. Before such a conclusion could be accepted, however, one should consider the stage of disease on admission in the three treatment groups (table 5). The numbers in certain groups are very small, but it is apparent that the streptomycin group was heavily weighted with cases of advanced disease (10 of 42, or 24 per cent) as compared with the other two groups (7 per cent and 10 per cent, respectively). It was shown above that calcification is commoner when there is delay in diagnosis (table 3). Combining the results in early and intermediate cases, the incidence of calcification was almost the same in the streptomycin and in the streptomycin-PAS group, 40 per cent and 38 per cent, respectively, showing that the addition of PAS to treatment made no difference. These two treatment groups can therefore be taken together and compared with the early and intermediate cases in the isoniazid group. This comparison shows that, of 69 patients who did not receive isoniazid, 28 (39 per cent) developed meningeal calcification as compared with only 12 of 43 patients (28 per cent) treated with isoniazid. Even these differences are not statistically significant. In addition, the isoniazid group had a shorter period of observation. Some children in this group may develop calcification in the future.

The effect of adjuvants on subsequent meningeal calcification: Intrathecal tuberculin was introduced as an adjuvant in the treatment of tuberculous meningitis by Smith and Vollum in 1950 (24). This method of treatment was subsequently adopted in the present series in selected children whose progress was unfavorable on routine antimicrobial treatment or whose disease was very advanced on admission. This group therefore was the least favorable of all. Fifteen such children survived for twenty-one months or longer, and only 5 of these had meningeal calcifications. These figures are small, but they suggest that intrathecal tuberculin may have been a factor in reducing the incidence of meningeal intracranial calcifications in this very ill group to a level below that of the over-all incidence of 37 per cent in the whole group of 130 patients. Streptokinase was given intrathecally during one period, and none of the 6 patients who received such treatment have any form of intracranial calcification. It is impossible, however, to draw conclusions from 6 cases.

Cerebral calcification: In 17 children (13 per cent) (tables 3, 4, and 5) calcification was detected in the brain substance. None of the calcification was in the cerebellum. In 15 cases the shadows were small, irregular in outline, and their diameter was only a few millimeters. It is likely that these shadows represented calcified "Rich foci." In 2 cases, however, the shadows were large, exceeding 10 mm. in diameter, and presumably represented large intracerebral tuberculomata.

FIG. 7.

FIG. 7A.

FIGS. 7, 7A; 8, 8A. Anterposterior and Towne views of same child as in figure 6, showing extension of calcification around the cisterna ambiens.

In one of these children the tuberculoma was situated in the left temporal lobe and was identified there by focal electroencephalographic changes as well as by roentgenograms. This child suffers from convulsions. His case has already been reported in greater detail earlier (16). The other child's lesion was in the occipital lobe (figures 10, 10A; 11, 11A). He is perfectly well and has a normal electroencephalogram.

FIG. 8.

FIG. 8A.

FIG. 9.

FIG. 9A.

FIG. 9, 9A. Calcification of the meninges in the Sylvian fissure. Normal child.

Three children who had intracerebral calcification also had meningeal calcification (figures 4, 4A; 5, 5A).

The incidence of intracerebral calcification was independent of the stage of the disease on admission or of the method of treatment (table 5).

The Incidence of Calcification in Children with Neurologic Sequelae

Among the 130 children there were 21 with varying degrees of neurologic sequelae (table 6). Thirteen of the 21 (62 per cent) had some form of intracranial calcification as compared with 50 (46 per cent) of the 109 children without

TABLE 3

INTRACRANIAL CALCIFICATION FOLLOWING TUBERCULOUS MENINGITIS IN RELATION TO THE STAGE ON ADMISSION

Minimal Follow-up: 2 Years

Stage on Admission	All Cases	Calcification					
		Basal		Cerebral		Total	
		Number	Per Cent	Number	Per Cent	Number	Per Cent
Early	33	7	21	5	15	12	36
Intermediate	79	32*	40	11*	14	40*	50
Advanced	18	10	55	1	5	11	61
Total	130	49*	37	17*	13	63	48

* Includes 3 patients with both types of calcification.

TABLE 4

INTRACRANIAL CALCIFICATION FOLLOWING TUBERCULOUS MENINGITIS IN RELATION TO METHOD OF TREATMENT

Minimal Follow-up: 2 Years

Treatment	All Cases	Calcification					
		Basal		Cerebral		Total	
		Number	Per Cent	Number	Per Cent	Number	Per Cent
Streptomycin	42	20*	48	7*	16	25*	60
Streptomycin and PAS	40	15	37	6	15	21	52
Streptomycin, PAS, and isoniazid	48	14*	29	4*	8	17*	35
Total	130	49*	37	17*	13	63*	48

* Includes patients with both types of calcification.

sequelae. These differences are not statistically significant. Sixteen of these 21 children are alive and 11 of them (68 per cent) have calcification. The only disability in one of these children is a flaccid weakness of one leg (*Case 14*). It is likely that this child, who has no intracranial calcification, had an attack of paralytic poliomyelitis while under treatment for tuberculous meningitis, because the course of the meningitis was wholly favorable until the unexpected occurrence of this paralysis. Apart from this child, the only children with neurologic sequelae who have no intracranial calcification are those who had intrathecal tuberculin treatment (*Cases 7*, *11*, *12*, and *15*). One of these has paraplegia, optic atrophy, sexual precocity, and mental retardation; another is a quadriplegic idiot; the third has hemiplegia; and the fourth is an idiot. These observations confirm the impression that intrathecal tuberculin treatment may be an effective method of dissolving meningeal exudate. They also show that the incidence of intracranial calcification may be higher in children with neurologic

TABLE 5

THE INCIDENCE OF INTRACRANIAL CALCIFICATION DETECTED DURING LIFE, IN RELATION TO STAGE ON ADMISSION AND TREATMENT

	Intracranial Calcification						Total Number of Patients
	None		Basal		Cerebral		
	Number	Per Cent	Number	Per Cent	Number	Per Cent	
Early							
Streptomycin	8		3		3		14
Streptomycin + PAS	8		1		2		11
Streptomycin + PAS + isoniazid	5		3		0		8
Total	21	64	7	21	5	15	33
Intermediate							
Streptomycin	7		10*		3*		18*
Streptomycin + PAS	9		13		4		26
Streptomycin + PAS + isoniazid	23		9†		4†		35†
Total	30	50	32‡	40	11‡	14	79‡
Advanced							
Streptomycin	2		7		1		10
Streptomycin + PAS	2		1		0		3
Streptomycin + PAS + isoniazid	3		2		0		5
Total	7	39	10	55	1	5	18
All stages							
Streptomycin	17	40	20*	48	7*	16	42*
Streptomycin + PAS	19	48	15	37	6	15	40
Streptomycin + PAS + isoniazid	31	65	14†	29	4†	8	48†
Total	67	51	49‡	37	17‡	13	130‡

* Includes 2 patients with cerebral and basal calcification.
† Includes 1 patient with cerebral and basal calcification.
‡ Includes 3 patients with cerebral and basal calcification.

sequelae, especially if they were not treated with intrathecal tuberculin. In view of the small numbers involved, however, the evidence for this statement is inadequate.

Late relapses and intracranial calcification: Six children in the present series suffered a late relapse of their tuberculous meningitis one to five years after their recovery, with normal cerebrospinal fluid. All of these children had basal meningeal intracranial calcification at the time of the relapse. Two made a good recovery after a fresh course of treatment, one is making good progress on treatment; but 3 died.

Necropsy Findings in Late Deaths

Among the 10 children who died twenty-one months or more after the onset of meningitis, there were 4 in whom no necropsy was performed. In 3 others no

TABLE 6

21 Children with Neurological Sequelae

Case Number	Age at Onset	Stage at Onset	Treatment Group	Intrathecal Tuberculin	Residual Lesion	Intracranial Calcification		Comments
						Basal	Cerebral	
a) Alive = 16								
	Years							
1	1	A	S	–	Hemiplegia, retarded ++	+	–	
2	2	I	S	–	Hemiplegia, retarded ++	++	–	
3	5	A	S	–	Optic atrophy, sexual precocity	+	–	
4	3	A	S	–	Hemiplegia	+	–	
5	5	E	S	–	Occasional convulsions	–	+	Reference 16
6	2	A	S	–	Paraplegia, ectopic ossifications	–	+	Lorber, 1953 (25)
7	2	A	S	+	Paraplegia, optic atrophy, sexual precocity, mental retardation; ectopic ossifications	–	–	Lorber, 1953 (25)
8	2	A	S	+	Optic atrophy with blindness, ament, sexual precocity, convulsions	++	–	
9	3	I	S	–	Hemiplegia	+	–	
10	9	A	S	–	Ament	+	–	
11	1	A	S + PAS	+	Quadriplegia, ament	–	–	
12	6	I	S + PAS + INH	+	Hemiplegia	–	–	
13	6	I	S + PAS + INH	–	Facial paralysis	+	–	
14	<1	E	S + PAS + INH	–	Flaccid paresis, right leg	–	–	? Poliomyelitis as complication
15	4	I	S + PAS + INH	+	Ament	–	–	
16	5	A	S + PAS + INH	+	Optic atrophy blind	+++	–	Figures 6, 7, 8
b) Dead = 5								
17	1	A	S	–	Hydrocephalus, ament	–	–	Died 26 months after onset
18	<1	A	S	–	Hydrocephalus, ament	+	–	Died 30 months after onset
19	2	A	S + PAS	+	Hydrocephalus, ament	–	–	Died 28 months after onset
20	5	I	S + PAS	+	Optic atrophy, blind	++		Figures 12 and 13; died 6 years after onset
21	1	I	S + PAS + INH	+	Hydrocephalus, ament	–	–	Died 39 months after onset

S = Streptomycin
INH = isoniazid
E = Early
I = Intermediate
A = Advanced

FIG. 10.

FIG. 10A.

FIGS. 10, 10A; 11, 11A. Lateral and Townes views, showing large cluster of areas of cerebral calcification in the right occipital lobe. There is a further solitary speck of calcification in the brain and another in the pineal gland. Normal child.

intracranial calcification was detected during life or at necropsy. In two other children no definite calcification was found during life, but at necropsy calcified and even ossified lesions were found. These have been described in detail in an earlier paper (1). Finally, there was one child who survived a severe attack of meningitis, but had bilateral optic atrophy with partial blindness. He remained well for five years and attended a school for the blind. Intracranial calcification was first detected two years after the onset of his meningitis; later the calcification became dense and massive. It occupied an area above and in front of the sella, in the position of the optic chiasma and the optic nerves (figure 12, 12A). The possibility of surgical removal of the calcified mass was being considered when the disease relapsed and the patient died in spite of treatment.

At necropsy a large calcified mass was found to occupy the whole of the interpeduncular cistern and the chiasmatic cistern. The optic tracts and the optic

FIG. 11.

FIG. 11A.

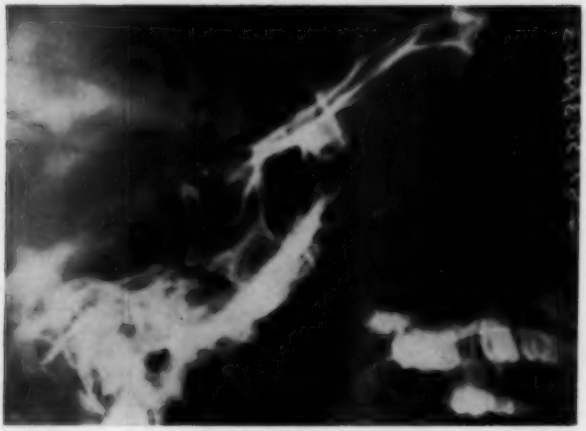

FIG. 12.

FIG. 12A.

FIG. 12, 12A. Extensive basal calcification, particularly involving the cisterna chiasmatis, but also extending further back. Blind child, (*Case 20*, table 6).

nerves were deeply embedded in the calcified mass and were not separately identifiable (figure 13). It was clear that surgical intervention at this stage could not have restored the boy's eyesight.

DISCUSSION

The present investigation confirms the very high incidence of intracranial calcification in children, following tuberculous meningitis. There is little reference to its incidence in adults, but in one series (13) of 50 adults followed for one to six years the incidence was only 2 per cent, although in the same series it was 26 per cent in 80 children. The proportion of children who develop intracranial calcification rises with increasing delay in the diagnosis and in the institution of adequate treatment. The incidence of meningeal calcification is higher in children who are left with permanent neurologic lesions, but the incidence is still high in children with no permanent complications whatever.

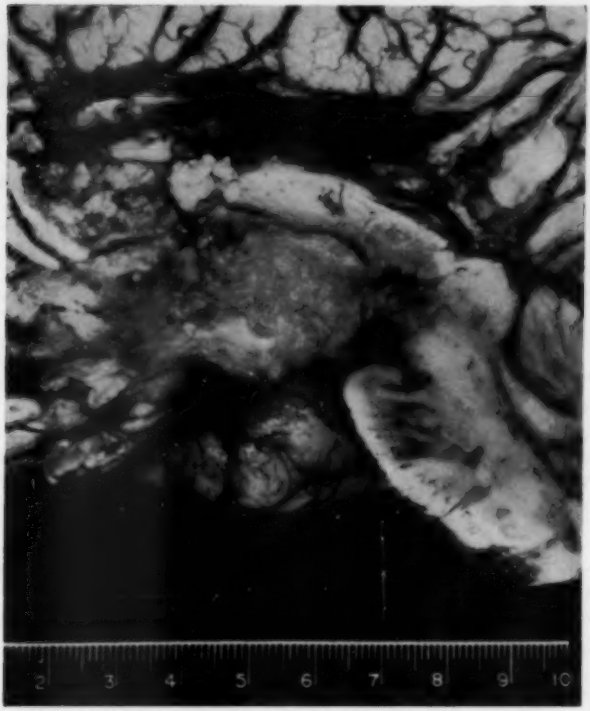

FIG. 13. Post-mortem photograph of the midbrain of the same child as in figure 12. He died during a relapse of the disease six years after his first attack of meningitis. Optic tract and optic nerves were completely embedded in extensive calcified exudate at the base of the brain.

There is some evidence that patients who have been treated with isoniazid develop calcification less often than those treated before isoniazid became available. This is true even when allowance is made for the differences in the composition of the groups in relation to the stage of the disease on admission. There was a low incidence of calcification in two small groups who were treated either with intrathecal tuberculin or intrathecal streptokinase. It will be of considerable interest to see whether the combination of the antimicrobial treatment with cortisone leads to a further reduction in the proportion of patients who develop intracranial calcification.

It appears that the presence of intracranial calcification is an unfavorable prognostic feature, because the 6 children in the present series who made a good recovery but suffered a late relapse of meningitis had intracranial calcification. It seems likely that these calcified masses may harbor living tubercle bacilli for years and that, under certain conditions, they may once more gain access to the meningeal spaces and set up fresh inflammation. Nevertheless, large numbers

of apparently normal children have lived for years with their calcified lesions without trouble. The present writer's first survivor was treated more than ten years ago. He still has both meningeal and cerebral calcification, but remains perfectly well.

As tuberculous meningitis is still a common disease in many parts of the world and as a substantial proportion of children recover on adequate treatment, it seems likely that this condition is becoming the most common cause of intracranial calcification in children.

Summary

Routine serial roentgenograms of the skull were taken in 130 children who recovered from tuberculous meningitis and who have been observed for two to ten years. One hundred and twenty of the children are alive at the time of the present report; 10 children died twenty-one months to six years after the onset of the meningitis.

Pathologic intracranial calcification was detected during life in 63 children (48.4 per cent). In 2 others who died, intracranial calcification and even ossification were found at necropsy. In most cases the calcification became detectable two to three years after the onset of the disease, but in some it was not detectable for five years.

The calcification occurred in two main sites: (*1*) the basal meninges and (*2*) within the brain substance. Calcification in the basal meninges was by far the more common and was seen in 49 cases (37 per cent). The appearance and distribution have been described in detail. The incidence of meningeal calcification progressively increased from 21 per cent in patients in the early stage of the disease on admission to 55 per cent in patients in the advanced stages. There was no appreciable difference in the incidence between those treated with streptomycin alone or with streptomycin-PAS, but the incidence of calcification was a little less in those treated with isoniazid-streptomycin. The use of adjuvants, intrathecal tuberculin and streptokinase, also reduced the incidence of meningeal calcification in selected groups.

Intracerebral calcification was seen in 17 children (13 per cent). The incidence was independent of the stage of the disease on admission and of the method of treatment.

The incidence of calcification was higher among 21 children with neurologic sequelae (62 per cent) than in the remaining 109 who had no such sequelae (46 per cent), but the differences are not significant. Intracranial calcified lesions may harbor living tubercle bacilli. All 6 children whose disease later relapsed had intracranial calcification.

It is considered likely that tuberculous meningitis is becoming the most common cause of intracranial calcifications in children.

Sumario

Calcificación Intracraneal Consecutiva a Meningitis Tuberculosa en los Niños

En 130 niños que se habían curado de meningitis tuberculosa y mantenidos en observación de dos a diez años, se tomaron radiografías seriadas corrientes del cráneo. Ciento veinte

se hallan vivos a esta fecha; 10 fallecieron de veintiún meses a seis años después de la iniciación de la meningitis.

En 63 niños (48.4 por ciento) se descubrió calcificación intracraneal patológica durante la vida. En otros 2 que fallecieron, se descubrieron calcificación y hasta osificación intracraneal en la autopsia. En la mayoría de los casos, la calcificación se volvió descubrible de dos a tres años después de la iniciación de la enfermedad, pero en algunos no pudo descubrirse por cinco años.

La calcificación apareció en dos sitios principales: (*1*) las meninges basales y (*2*) dentro de la substancia cerebral. El primero fué con mucho el más frecuente, observándose en 49 casos (37 por ciento). Descríbense con todo pormenor el aspecto y la distribución. La incidencia de calcificación meníngea aumentó progresivamente de 21 por ciento en los enfermos en el período incipiente de la enfermedad a su ingreso a 55 por ciento en los enfermos del período avanzado. No hubo diferencia apreciable de la incidencia entre los tratados con estreptomicina sola o con estreptomicina-PAS, pero la incidencia fué un poco menor en los tratados con isoniacida-estreptomicina. El empleo de coadyuvantes, de tuberculina y estreptoquinasa por vía intratecal, también rebajó la incidencia de calcificación intracraneal en grupos escogidos.

Se observó calcificación intracerebral en 17 niños (13 por ciento). La incidencia fué independiente del período de la enfermedad al ingreso y del método de tratamiento.

La incidencia de calcificación fué más alta (61 por ciento) entre 21 niños que tuvieron secuelas neurológicas que entre los 109 restantes que no tuveron tales secuelas (46 por ciento), pero las diferencias no fueron importantes. Las lesiones calcificadas intracraneales pueden albergar bacilos tuberculosos vivos. Los 6 niños que experimentaron recidivas tardías tenían calcificación intracraneal.

Se considera probable que la meningitis tuberculosa se va volviendo la causa más común de las calcificaciones intracraneales en los niños.

Resume

La calcification intracranienne consécutive à la méningite tuberculeuse chez l'enfant

Des radiogrammes systématiques du crâne ont été pratiqués chez cent-trente enfants guéris d'une méningite tuberculeuse et maintenus en observation pendant deux à cinq ans. Au moment du rapport actuel, 120 d'entre eux sont vivants; 10 sont morts entre vingt-et-un mois et six ans après le début de la méningite.

La calcification pathologique intracranienne a été décelée durant la vie chez 63 enfants (48, 4%). Chez 2, la calcification et même l'ossification intracranienne n'ont été découvertes qu'à l'autopsie. Dans la plupart des cas la calcification a été visible deux à trois ans après le début de la maladie; dans les autres, elle n'a été décelée qu'au bout de cinq années.

La calcification s'est localisée en deux points principaux: (*1*) les méninges de la base; (*2*) la substance cérébrale. La localisation au niveau des méninges de la base était la plus fréquente et a été observée dans 49 cas (37%). L'aspect et la distribution de la calcification ont été décrits en détail. L'incidence de la calcification méningée progressive s'est élevée de 21%, chez les malades admis à l'hôpital au stade initial de la maladie, à 55% chez les malades admis au stade avancé. Il n'y avait pas de différence appréciable dans l'incidence entre les cas traités par la streptomycine ou par l'association PAS-streptomycine, mais l'incidence de la calcification était légèrement inférieure dans les cas qui avaient été traités par l'association isoniazide-streptomycine. L'usage d'adjuvants, tuberculine et streptokinase, a également réduit l'incidence de la calcification méningée dans les groupes sélectionnés.

La calcification intra-cérébrale a été décelée chez 17 enfants (13%). Cette incidence était sans relation avec le stade de l'affection au moment de l'admission à l'hôpital et la méthode de traitement.

L'incidence de la calcification était plus élevée chez 21 enfants porteurs de séquelle neurologiques (62%) que chez ceux qui en étaient indemnes (46%), mais ces différences ne

sont pas significatives du point de vue statistique. Les lésions calcifiées intracraniennes peuvent recéler des bacilles tuberculeux vivants. Chez les 6 enfants qui ont présenté des rechutes tardives, il existait des calcifications intracraniennes.

L'auteur estime qu'il est probable que c'est la méningite tuberculeuse qui devient la cause la plus fréquente des calcifications intracraniennes chez l'enfant.

Acknowledgments

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A shorter version of this paper was delivered at the 1st International Congress of the Neurological Sciences in July, 1957, in Bruxelles and the writer wishes to thank the Board of Governors of the United Sheffield Hospitals for their grant to enable him to attend the Congress.

Addendum

Acheson and Smith (26) published a paper after the present study was completed. In that paper, intracranial calcification was reported in 14 per cent of 104 cases of tuberculous meningitis which had been followed for at least two years after commencement of treatment. The 104 patients were mostly adults. The writers agreed that calcification was more common in children than in adults and that it was more likely to form in severe than in mild cases. They confirmed that it was less often found in patients who received intrathecal tuberculin as an adjuvant to treatment.

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ANTITUBERCULOSIS ACTIVITY IN THE PHENAZINE SERIES[1]

II. N^3-Substituted Anilino*apo*safranines (Rimino-Compounds) and Some Derivatives

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INTRODUCTION

In a previous report (1), the writers have described the antituberculous activity in mice and guinea pigs of anilino*apo*safranines and their isomers. Further synthetic work has led to the rimino-compounds (2), and a preliminary account (3) of their biologic properties has already been published. These compounds may be considered as being derived from the anilino*apo*safranines by substitution of the hydrogen atom of the imino-group in the 3-position of the phenazine nucleus, or from their isomers by substitution of a hydrogen atom of the amino-group in the 2-position of the phenazine nucleus.

The present communication describes the antituberculous activity in mice of 67 rimino-compounds, 11 glyoxalinophenazines (4), and a small number of *apo*safranones (5). All of these structures, I–VI, are shown in figure 1. The results of some *in vitro* experiments are also recorded.

MATERIALS AND METHODS

The experimental methods have previously been described in detail (6). In brief, *in vitro* activity against *M. tuberculosis* H37Rv was estimated in a Proskauer and Beck medium and in the same medium to which human serum (5 per cent) had been added. For determinations of *in vivo* activity, groups of 10 albino mice (Schofield) were infected intravenously with 0.1 mg. (moist weight) of the bovine Ravenel Rv strain of *M. tuberculosis* grown for nine to twelve days in a Dubos liquid medium. Drugs were administered in a ground biscuit diet for fourteen days, commencing on the day of infection or on the sixth day of the disease. In some experiments an isoniazid-resistant variant of the Ravenel Rv strain was used. Every mouse which died was examined to ascertain the cause of death, and the criterion of antituberculous activity was a significant increase (19 of 20 probability, or better) in the median survival time of the treated mice over the controls. The median survival time of untreated control mice was approximately fifteen to eighteen days throughout the series of experiments.

OBSERVATIONS

Antituberculous Activity in Mice

As may be seen in table 1, of the 67 compounds of *type III* examined, only 2, at the doses recorded, are without activity in murine tuberculosis. There is a considerable variation in activity, however, in the range of compounds examined. When $R' = R''' = Ph$ and R'' represents straight chain alkyl groups (CH_3 to n-$C_{12}H_{25}$), maximal activity is encountered with the compounds B.760 (R'' = n-butyl) and B.783 (R'' = n-amyl). A similar pattern is repeated when R'' is a

[1] From the Laboratories of the Medical Research Council of Ireland, Trinity College Dublin.

[2] See reference (1) for Part I.

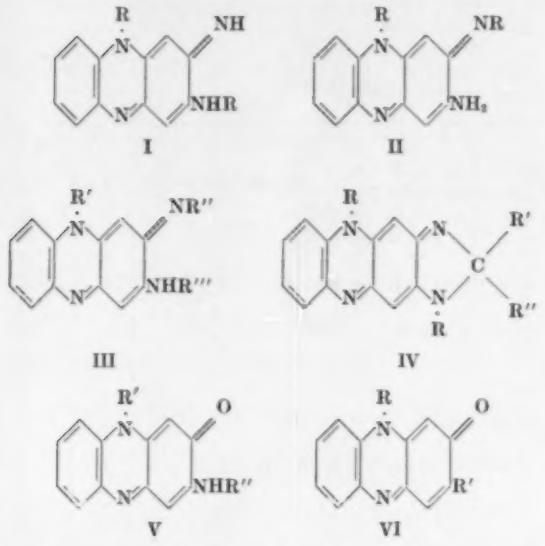


FIG. 1. I. Anilino*apo*safranines; II. isomers of *Type I*; III. rimino compounds; IV. glyoxalinophenazines; V and VI. *apo*safranones.

branched alkyl chain, although the activity in this group is higher. Maximal activity is found here with B.775 (R″ = *iso*butyl). The aralkyl derivative, B.751 (R″ = phenethyl) is about equal in activity to that of B.779 (R″ = *n*-propyl). The R″ *cyclo*alkyl derivatives may be considered also as having branched-chain substituents, and, as might be expected, the best of them, B.669, shows the same high order of activity.

In another group of compounds (B.664, B.778, B.800, B.803, B.720, and B.805) R″ represents an alkyl group carrying a hydrophilic substituent. With the exception of B.720 (R″ = diethylaminoethyl), these compounds have lower activity than the parent alkyl derivatives. Again when the R″ substituent is aromatic (B.710 and B.726), the activity is moderate.

The compounds B.283 (anilino*apo*safranine) and the dichloro-derivative, B.628, although not, by definition, rimino-compounds, have been included in table 1 for purposes of comparison. The potentiating effect of two chlorine atoms substituted in the periphery of the molecule as in B.628 has already been noted (1). Chlorine substitution has a similar effect on the activity of the rimino-compounds. In this group the same pattern of activity was found as in the unchlorinated derivatives, but at a much higher level. Thus, when R″ = *n*-alkyl, a plateau activity is shown when the chain contains 2 to 5 carbon atoms; with less or more carbon, there is a sharp falling off.

Again when the carbon chain of R″ is branched, highest activity is found with 2 to 5 carbons in R″. As before, the branching may be supplied by *cyclo*alkyl

TABLE 1

ACTIVITY IN MURINE TUBERCULOSIS OF RIMINO-COMPOUNDS ADMINISTERED IN THE DIET

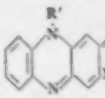
Number		Type of Test	Intake of Drog Daily	Change in Median Survival Time
			mg/kg	*days*
B.283	R′ = R‴ = Ph; R″ = H (Anilinoaposafranine)	P	70	+7.5
		P	50	inactive
B.717	R′ = R‴ = Ph; R″ = CH_3	P	50	inactive
B.718	R′ = R‴ = Ph; R″ = C_2H_5	P	20	+5
		E	85	+10
B.779	R′ = R‴ = Ph; R″ = *n*-C_3H_7	P	20	+16
		P	5	inactive
B.670	R′ = R″ = Ph; R″ = ·$CH(CH_3)_2$	P	20	+27
		P	5	inactive
		E	170	+66
		E	10	+6
B.760	R′ = R‴ = Ph; R″ = *n*-C_4H_9	P	5	+5
		E	10	+17
B.775	R′ = R‴ = Ph; R″ = ·CH_2·$CH(CH_3)_2$	P	5	+22
		E	10	+35
B.688	R′ = R‴ = Ph; R″ = $CH(CH_3)C_2H_5$	P	5	+8
		E	135	+60
		E	10	+9
B.783	R′ = R‴ = Ph; R″ = *n*-C_5H_{11}	P	5	+5
		E	10	+25
B.687	R′ = R‴ = Ph; R″ = ·$CH(C_2H_5)_2$	P	5	+4
		E	10	+5
B.690	R′ = R‴ = Ph; R″ = ·$CH(CH_3)$·CH_2·$CH(CH_3)_2$	P	20	+30
		P	5	inactive
		E	200	+70
B.719	R′ = R‴ = Ph; R″ = *n*-C_7H_{15}	P	20	+17
		P	5	inactive
B.715	R′ = R‴ = Ph; R″ = ·C_2H_4·$CH(CH_3)$·CH_2·$C(CH_3)_2$	P	60	+50
		P	20	inactive
		E	80	+43
		E	10	inactive
B.721	R′ = R‴ = Ph; R″ = *n*-$C_{10}H_{21}$	P	20	inactive
		E	10	inactive
B.722	R′ = R‴ = Ph; R″ = *n*-$C_{12}H_{25}$	P	20	inactive
B.796	R′ = R‴ = Ph; R″ = *cyclo*pentyl	P	20	+35
		P	5	+6
		E	10	+5
B.669	R′ = R‴ = Ph; R″ = *cyclo*hexyl	P	100	+135
		P	5	+20
		P	2	inactive
		E	155	+125
		E	10	+34
B.730	R′ = R‴ = Ph; R″ = 4-methyl*cyclo*hexyl	P	5	+10
		E	10	+18
B.737	R′ = R‴ = Ph; R″ = 3,5-dimethyl*cyclo*hexyl	P	20	+40
		P	5	inactive

TABLE 1—CONTINUED

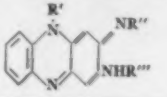
Number		Type of Test	Intake of Drug Daily	Change in Median Survival Time
			mg/kg	*days*
B.729	R′ = R‴ = Ph; R″ = *cyclo*heptyl	P	5	+7
		E	10	+30
B.664	R′ = R‴ = Ph; R″ = ·CH_2OH	P	50	+9
		P	20	inactive
B.778	R′ = R‴ = Ph; R″ = ·C_2H_4OH	P	20	+7
		P	5	inactive
B.805	R′ = R‴ = Ph; R″ = ·$C_2H_4OCH_3$	P	50	+12
		P	20	+3
B.800	R′ = R‴ = Ph; R″ = ·*n*-C_3H_6OH	P	50	+7
		P	20	inactive
B.803	R′ = R‴ = Ph; R″ = ·$C_2H_4NH_2$	P	50	+15
		P	20	inactive
B.720	R′ = R‴ = Ph; R″ = ·$C_2H_4N(C_2H_5)_2$	P	20	+76
		P	5	+30
		E	10	inactive
B.710	R′ = R‴ = Ph; R″ = Ph	P	20	+30
		P	5	inactive
		E	70	+30
		E	10	inactive
B.726	R′ = R‴ = Ph; R″ = ·C_6H_4Cl	P	20	+12
		P	5	inactive
B.751	R′ = R‴ = Ph; R″ = ·$(CH_2)_2$·Ph	P	20	+12
		P	5	inactive
B.628	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = H	P	120	+117
		P	20	+75
		P	5	+32
		E	10	+17
B.743	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = CH_3	P	5	+9
		E	10	inactive
B.746	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = C_2H_5	P	5	+45
		E	10	+60
B.777	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = *n*-C_3H_7	P	5	+38
		E	10	+82
B.663	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = ·$CH(CH_3)_2$	P	20	>+300
		P	5	+210
		P	2	+95
		P	1	+15
		E	180	+400
		E	10	+180
		E	5	+90
		E	2	+9
B.759	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = *n*-C_4H_9	P	5	+50
		E	10	+43
B.776	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = ·CH_2·$CH(CH_3)_2$	P	5	+125
		E	10	+120
B.761	R′ = R‴ = ·C_6H_4Cl(*p*); R″ = ·$CH(CH_3)$·C_2H_5	P	5	+110
		E	10	+110

TABLE 1—CONTINUED

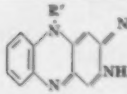
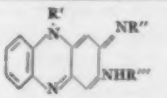
Number		Type of Test	Intake of Drug Daily	Change in Median Survival Time
			mg/kg	*days*
B.784	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *n*-C_5H_{11}	P	5	+45
		E	10	+45
B.762	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·$CH(C_2H_5)_2$	P	5	+90
		E	10	+110
B.755	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *n*-C_6H_{13}	P	20	+40
		P	5	+3
		E	10	inactive
B.754	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *n*-$C_{10}H_{21}$	P	20	+6
		P	5	inactive
B.752	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *n*-$C_{12}H_{25}$	P	20	+5
		P	5	inactive
B.797	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *cyclo*pentyl	P	5	+75
		E	10	+75
B.673	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *cyclo*hexyl	P	5	+145
		P	2.5	+30
		E	10	+200
		E	5	+120
B.741	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = 4-methyl-*cyclo*hexyl	P	5	+95
		E	10	+120
B.742	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = 3,5-dimethyl-*cyclo*hexyl	P	5	+48
		E	10	+65
B.740	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = *cyclo*heptyl	P	5	+100
		E	10	+120
		E	5	+115
B.782	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·C_2H_4OH	P	5	+8
		E	10	+30
B.806	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·$C_2H_4OCH_3$	P	20	+27
		P	5	+16
		E	10	+27
B.804	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·$C_2H_4NH_2$	P	20	+19
		P	5	inactive
		E	10	inactive
B.799	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·*n*-C_3H_6OH	P	20	+33
		P	5	+3
		E	10	inactive
B.749	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·$C_2H_4N(C_2H_5)_2$	P	5	+20
		E	10	+65
B.725	R′ = R‴ = ·$C_6H_4Cl(p)$; R″ = ·$C_6H_4Cl(p)$	P	20	+3
		P	5	inactive
B.750	R′ = Ph; R″ = R‴ = *cyclo*hexyl	P	20	+20
		P	5	inactive
B.731	R′ = Ph; R″ = R‴ = C_6H_4·$CH_2(p)$	P	20	+14
		P	5	inactive
B.732	R′ = Ph; R″ = R‴ = ·$C_6H_4Cl(p)$	P	20	+5
		P	5	+3
B.738	R′ = R″ = R‴ = ·$C_6H_4OCH_3(p)$	P	20	+25
		P	5	inactive

TABLE 1—CONTINUED

Number		Type of Test	Intake of Drug Daily	Change in Median Survival Time
			mg/kg	
B.753	R′ = ·$C_6H_4Cl(p)$; R″ = R‴ = *cyclo*hexyl	P	5	+30
		E	10	+45
B.739	R′ = ·$C_6H_4Cl(p)$; R″ = R‴ = Ph	P	20	+17
		P	5	inactive
B.744	R′ = CH_3; R″ = R‴ = Ph	P	20	+7
		P	5	inactive
B.745	R′ = CH_3; R″ = R‴ = ·$C_6H_4Cl(p)$	P	5	+11
		E	10	+26
B.733	R′ = *cyclo*hexyl; R″ = R‴ = Ph	P	20	+6
		P	5	inactive
B.734	R′ = *cyclo*hexyl; R″ = R‴ = $C_6H_4Cl(p)$	P	50	+6
		P	20	inactive
		E	10	inactive
B.756	R′ = ·$C_2H_4N(C_2H_5)_2$; R″ = R‴ = Ph	P	5	+9
		E	10	+10
B.757	R′ = ·$C_2H_4N(C_2H_5)_2$; R′ = R‴ = ·$C_6H_4Cl(p)$	P	20	+42
		P	5	inactive
B.788	R′ = R‴ = ·$C_6H_4OCH_3(p)$; R″ = ·$CH(CH_3)_2$	P	20	+27
		P	5	inactive
B.787	R′ = R‴ = ·$C_6H_4OCH_3(p)$; R″ = *cyclo*hexyl	P	20	+17
		P	5	inactive
B.790	R′ = R‴ = ·$C_6H_4OCH_3(p)$; R″ = *cyclo*hexyl	P	5	+6
		E	10	+20
B.758	R′ = R‴ = ·$C_6H_4Cl(o)$; R″ = ·$CH(CH_3)_2$	P	20	+22
		P	5	inactive

P = protective type of test.
E = treatment delayed until six days after infection.

substituents. Thus, of the former type, B.663 (R″ = *iso*propyl), B.776 (R″ = *iso*butyl), B.761 (R″ = *sec*butyl), and B.762 (R″ = *sec*amyl); and, of the latter type, B.797 (R″ = *cyclo*pentyl), B.673 (R″ = *cyclo*hexyl), B.741 (R″ = 4-methyl*cyclo*hexyl), and B.740 (R″ = *cyclo*heptyl) all exhibit very high activity in the test at a dosage level of 5 mg. per kg. per day in a protective type of experiment and at 10 mg. per kg. per day in established disease, i.e., a six-day-old infection. On the other hand, it is interesting that B.758, the isomer of B.663, in which the chlorine atoms are *ortho*-substituted, has a much lower activity.

The introduction of chlorine is not of itself sufficient to confer high activity. Thus B.725, in which R′, R″ and R‴ are all *p*-chlorophenyl residues, is a much less effective compound than the chlorine-free analogue B.710 (R′ = R″ = R‴ = phenyl), from which it is derived. The compound, B.732, which has *p*-chlorophenyl substituents in the R″ and R‴ positions only, has again the lower activity of B.725, and both B.725 and B.732 are less active than B.739

which has only one *p*-chlorophenyl group (at R′), R″ and R‴ both being phenyl in this compound. None of the compounds in which all of the substituents R′, R″ and R‴ are aromatic shows significant activity at a dose of 5 mg. per kg., although some of them (B.731, B.732, B.738, and B.739) are active at the higher dose of 20 mg. per kg. Furthermore, replacement of chlorine in the phenyl residues by methoxyl makes for a reduction in activity (*cf.* the pairs, B.663 and B.788, or B.673 and B.787). When the chlorine is replaced by ethoxyl (B.790), the same relationship holds although B.790 in contrast to B.787 does show significant activity at 5 mg. per kg.

Those compounds in which R′ is nonaromatic in character form an interesting group (B.744, B.745, B.756, B.757, B.733, and B.734). In these six compounds, R″ and R‴ are phenyl or *p*-chlorophenyl residues. Of the compounds containing chlorine, only B.745 (R′ = methyl); and of those without chlorine, only B.756 (R′ = diethylaminoethyl) show activity at a dose of 5 mg. per kg. In the compounds, B.750 and B.753, R″ = R‴ = *cyclo*hexyl, and the R′ substituent is either phenyl or *p*-chlorophenyl. Only in the latter case (B.753) is activity found at 5 mg. per kg., showing the effect of this chlorine substituent. It would be of interest to examine a series of compounds containing *p*-chlorophenyl substituents in R′ and R″, but a synthetic route to compounds of this kind has not so far been found.

The best of the compounds in table 1 are more active in the test than any other compounds hitherto described. For example, B.663 (molecular weight = 473), in a protective type of experiment at a dose of 1 mg. per kg. shows significant activity. On a molar basis this would correspond to a dose of somewhat less than 0.3 mg. per kg. of isoniazid (molecular weight = 137), a dosage level at which the latter compound is inactive. The marked activity of B.663 in an established infection is also apparent.

A feature in some experiments, particularly when minimally effective doses were employed, was the occasional death from tuberculosis in the treated groups within the period covered by the deaths of the controls, the remaining animals surviving for long periods.

As previously reported, B.663 has marked curative properties in established tuberculosis in guinea pigs (3). At a dosage level of about 100 mg. per kg. in both guinea pigs and mice, it was noted that a crystalline deposit was found in various organs of the animals at autopsy. That these crystals were of unchanged material has now been confirmed by extraction from the tissues and by carrying out melting-point and mixed-melt determinations.

This phenomenon of crystal deposition prompted the carrying out of prophylactic experiments, the results of one of which may be seen in table 2. Mice which were fed the drug in the diet at a level of 10 mg. per kg. per day for fourteen days and were then taken off treatment are afforded a considerable measure of protection against the usual infecting inoculum given four weeks later. At a smaller dose (2 mg. per kg.), protection is evident after a one-week but not after a four-week interval. It is worth noting that, while all of the untreated control mice had died by the twentieth day, the first mouse, which was fed at 10 mg. per

TABLE 2

CHEMOPROPHYLAXIS IN MICE WITH THE PHENAZINE DERIVATIVE B.663*

(Ravenel Rv 0.1 Mg. Intravenously)

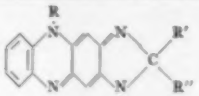
Dosage for 14 days	Interval Between End of Treatment and Day of Infection	Deaths from Tuberculosis
mg/kg/day	*weeks*	*days of deaths*
10	4	18, 25, 37, 51, 74, 80, 81, 83, 85, 98
10	1	61, 80, 82, 93, 98, 99, 104, 108, 140†, 140†
2	4	14, 14, 15, 15, 15, 17, 20, 20, 21, 41†
2	1	16, 16, 17, 19, 21, 21, 25, 26, 28, 41†
0		13, 14, 14, 14, 15, 16, 16, 18, 19, 20

* Mice from one batch were started on drug at appropriate intervals. All were infected on the same day with 0.2 ml. of the same Ravenel Rv suspension.

† Killed.

TABLE 3

ACTIVITY OF GLYOXALINO-PHENAZINES IN MURINE TUBERCULOSIS

Number		Type of Test	Intake of Drug Daily	Change in Median Survival Time
			mg/kg	*days*
B.682	R = *cyclo*hexyl; R′ = R″ = CH_3	P	200	inactive
B.621	R = Ph; R′ = R″ = CH_3	P	70	+8
B.675	R = ·$C_6H_4CH_3$(*p*); R′ = R″ = CH_3	P	200	inactive
B.654	R = ·C_6H_4Cl(*p*); R′ = R″ = CH_3	P	110	+13
B.665	R = Ph; R′ = CH_2Cl; R″ = CH_3	P	95	inactive
B.635	R = Ph; R′ = CH_3; R″ = C_2H_5	PR	135	+13
B.686	R = Ph; R′ = R″ = C_2H_5	P	200	inactive
B.649	R = Ph; CR′ R″ = *cyclo*hexane	PR	110	+5
B.650	R = ·C_6H_4·$OCH(CH_3)_2$; CR′ R″ = *cyclo*-hexane	PR	115	+18
B.672	R = ·C_6H_4Cl(*p*); CR′ R″ = *cyclo*hexane	P	80	+50
B.693	R = Ph; R′ = CH_3; R″ = 4-pyridyl	P	200	inactive

P = protective type of test.

PR = infected with Ravenel strain, using isoniazid-resistant Ravenel strain.

kg. and taken off treatment one week before infection, died on the sixty-first day, and 2 were still alive on the one hundred fortieth day when the experiment was terminated.

This is a true chemoprophylaxis and, as far as the present writers are aware it has not been achieved hitherto in animal tuberculosis with any other chemical

TABLE 4

ACTIVITY AGAINST M. TUBERCULOSIS H37Rv IN VITRO

(14 Days' Incubation)

Number	In Proskauer and Beck Medium	In Proskauer and Beck Medium Enriched with Serum
B.717	I 0.2	I 1.0
	F 0.05	F 0.13
B.743	I 0.1	I 1.0
	F 0.01	F 0.13
B.777	I 0.5	I 0.66
	F <0.01	F 0.02
B.670	I 0.3	I 1.0
	F 0.03	F 0.05
B.663	I 0.3	I 1.3
	F 0.06	F 0.2
B.775	I 0.13	I 1.0
	F 0.02	F 0.03
B.776	I 0.13	I 0.5
	F 0.05	F 0.02
B.719	I 0.06	I 0.6
	F 0.02	F 0.13
B.782	I 0.10	I 0.66
	F 0.02	F 0.05
B.721	I 0.66	I 3.3
	F 0.05	F 0.2
B.754	I 1.33	I 2.0
	F 0.06	F 0.33
B.669	I 0.06	I 0.66
	F 0.01	F 0.20
B.673	I 0.5	I 0.33
	F <0.01	F 0.05
B.740	I 0.13	I 0.5
	F 0.01	F 0.03
B.804	I 0.2	I 1.3
	F 0.05	F 0.66
B.725	I 2.5	I 3.3
	F 0.06	F 2.5

I = inhibitory concentration in γ per ml.

F = concentration in γ per ml., allowing full growth as in control cultures.

agent. The effect was obtained with treatment regimens which did not result in crystal deposition in the tissues although some pigmentation of the tissues was evident. It is suspected that the property of deposition in the tissues belongs to these rimino-compounds as a group, but so far prophylactic experiments have not been extended to any other of these compounds.

As might be expected, tubercle bacilli resistant to the usual antituberculous agents are susceptible to the rimino-compounds. As will be described in a subsequent report, it has not been possible to produce a variant of the H37Rv strain resistant to B.663 after seventeen subcultures. Also bacilli of the Ravenel Rv strain recovered from mice as long as 470 days after cessation of treatment with B.663 (180 mg. per kg. per day for fourteen days) showed no change in susceptibility to the drug.

The glyoxalinophenazines in table 3 show a moderate activity; they have not been extensively investigated. The following *apo*safranone derivatives, B.678 (VI. R′ = H; R = $C_6H_4Cl(p)$), B.684 (V.R′ = Ph; R″ = $\cdot C_6H_4Cl(p)$), B.685 (V.R′ = R″ = $\cdot C_6H_4Cl(p)$), and B.691 (V.R′ = $\cdot C_6H_4Cl(p)$; R″ = *cyclo*hexyl) were all inactive when fed in the diet at 200 mg. per kg. in a protective type of experiment.

The rimino-compounds are bland substances, well tolerated by mice, guinea pigs, and rabbits on oral administration. Their pharmacology will be dealt with in a further communication.

In table 4 may be seen the concentrations of a number of selected rimino-compounds which completely inhibit visible growth of *M. tuberculosis* H37Rv. There is no significant relationship between *in vitro* and *in vivo* activity, compounds differing greatly in their ability to protect mice having approximately the same inhibitory concentrations. The inhibitory dilutions are in all cases reduced in the presence of serum, but here also it has not been possible to relate serum antagonism to *in vivo* activity. The glyoxalinophenazines were so insoluble in various media that no reliable *in vitro* evaluation was possible.

Summary

Sixty-seven phenazine derivatives (rimino-compounds) have been examined in experimental tuberculosis in mice. Eight compounds showed a very high order of activity, in both the protective type of experiments and when the infection had been allowed to progress for six days before treatment was started.

The most thoroughly investigated phenazine derivative, B.663, showed significant antituberculous activity in mice at a dosage level of 1 mg. per kg. per day. When fed at a level of 100 mg. per kg. per day to mice for fourteen days, a crystalline deposit of B.663 was found in various organs at autopsy.

Mice fed 10 mg. per kg. of the phenazine derivative B.663 for fourteen days and infected four weeks later were substantially protected. This chemoprophylaxis was observed with dosage regimens which do not result in crystal deposition in the tissues.

Seventeen successive subcultures of H37Rv bacilli in the presence of subin-

hibitory concentrations of the B.663 derivative failed to produce any change in susceptibility to the drug.

The phenazine derivative B.663 has very low toxicity for mice, guinea pigs, and rabbits.

Sumario

Actividad Antituberculosa en la Serie de la Fenacina. Parte II. Anilinoaposafraninas con Substitución de N³ (Compuestos Riminicos) y Algunos Derivados

Sesenta y siete derivados de la fenacina (compuestos rimínicos) han sido estudiados en la tuberculosis murina. Ocho de ellos revelaron actividad de orden muy alto en experimentos con la *enfermedad protectora* y *establecida*.

El compuesto investigado más a fondo, B.663, desplegó actividad importante en los ratones a un nivel posológico de 1 mg. por kg. al día. Cuando se sumnistró a un nivel alto de 100 mg. por kg. al día a los rat tones durante catorce días, se observó un depósito cristalino de B.663 en varios óragnos en la autopsia.

Los ratones a los que suministraron 10 mg. por kg. de B.663 durante catorce días quedaron protegidos substancialmente. Esta quimioprofilaxis fué observada con regímenes posológicos que no ocasionaron depósitos de cristales en los tejidos.

Diecisiete subcultivos sucesivos de bacilos H37Rv en presencia de concentraciones subinhibidoras de B.663 no produjeron la menor alteración de la susceptibilidad a la droga.

El B.663 posee muy poca toxicidad para los ratones, cobayos y conejos.

Resume

Activité antituberculeuse dans la série phénazine. II. N3-substitué anilinoaposafranines (composés rimino) et quelques dérivés

On a étudié dans la tuberculose de la souris 67 dérivés de la phénazine (composés rimino). Huit d'entre eux ont présenté en haut degré d'activité dans l'expérimentation à l'égard de la *prophylaxie* et de la *maladie établie*.

Le composé qui a été le plus extensivement étudié, le B.663, a révélé une activité notable chez la souris à la dose de 1 mg/kg par jour. Administré à la dose élevée de 100 mg/kg par jour à la souris pendant quatorze jours, on a retrouvé à l'autopsie un dépôt de cristaux de B.663 dans divers organes.

Les souris recevant 10 mg/kg de B.663 pendant quatorze jours et inoculées quatre semaines plus tard ont été substantiellement protégées. Cette chimioprophylaxie a été observée avec des schémas posologiques qui n'ont pas provoqué le dépôt de cristaux dans les tissus.

Dix-sept sous-cultures en série de bacilles H37Rv en présence de concentration subinhibitrices de B.663 n'ont pas réussi à modifier la sensibilité au médicament.

Le B.663 présente une toxicité réduite chez la souris, le cobaye et le lapin.

Acknowledgments

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THE EFFECT OF 5-HEPTYL-2-THIOHYDANTOIN ON EXPERIMENTAL TUBERCULOSIS[1]

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Introduction

Of the large number of substances tested for antituberculous activity by research laboratories throughout the world, very few have exhibited the potency and safety required for an antituberculous drug. Great advances have been made in the fields of chemotherapy and management of tuberculosis during the last decade, but poor tolerance of a drug by certain patients, undesirable or toxic side effects, and particularly the emergence of drug-resistant tubercle bacilli during the course of prolonged therapy are factors which sustain active search for new and better antituberculous agents.

During the course of "screening" chemical compounds for antituberculous activity in these laboratories, it was found that 5-hexyl-2-thiohydantoin (1) exhibited a significant therapeutic effect when tested in mice infected with *M. tuberculosis*, strain H37Rv. This observation stimulated further investigation of the antituberculous activity of thiohydantoin derivatives. A preliminary report of these studies has been published elsewhere (2). After testing a variety of derivatives of this compound (3), it was shown that antituberculous activity was limited to 5-alkyl-2-thiohydantoins and that maximal effectiveness was attained with 5-heptyl-2-thiohydantoin, designated Win 5211 (figure 1). The activity decreased precipitously as the 5-alkyl chain was lengthened or shortened, and numerous homologues and other related compounds were shown to be without any appreciable antituberculous activity.

Data will be presented in this report concerning the antituberculous activity of 5-heptyl-2-thiohydantoin (Win 5211), as determined by laboratory studies in experimental animals. These studies include therapeutic experiments in mice and hamsters infected with various strains of tubercle bacilli of human origin and similar experiments in mice infected with bovine tubercle bacilli.

Materials and Methods

Bacterial strains: The H37Rv human strain and the Ravenel bovine strain of *M. tuberculosis* were originally obtained from Dr. William Steenken, Jr., Trudeau Laboratory, Saranac Lake, New York. Isoniazid-resistant and streptomycin-resistant variants of the H37Rv strain were obtained from Dr. Charles Hurwitz, Veterans Administration Hospital, Albany, New York. All cultures were maintained in Proskauer-Beck liquid medium. Virulence was maintained by periodic passages through mice and guinea pigs.

Female Swiss albino mice, Webster strain, 14 to 16 gm. of body weight, obtained from Taconic Farms, were used in all experiments.

Infection procedure: The animals were infected intravenously with 0.1 mg. (H37Rv) or 1.5 mg. (isoniazid- and streptomycin-resistant variants), or 0.05 mg. (Ravenel) of mycobacteria, in terms of moist weight. The inoculum was prepared from two-week-old cultures

[1] From Sterling-Winthrop Research Institute, Rensselaer, New York.

by suspending the proper amount of tubercle bacilli in 0.1 ml. of Dubos' Tween®-albumin liquid medium.

Medication: Medication was started three days after infection and was continued for twenty eight days. As the thiohydantoin (Win 5211) is relatively insoluble in water, it was administered to mice as a suspension in 8 per cent autoclaved gelatin by stomach tube twice daily, five days a week, at doses of 150, 300, and 600 mg. per kg. per day. In the experiments with isoniazid-resistant and streptomycin-resistant tubercle bacilli, additional groups of mice were medicated with isoniazid or dihydrostreptomycin, respectively, as an index of resistance. Included in each experiment was an infected and nontreated control group of mice. The experiment was terminated arbitrarily on the thirty-first day of infection when all surviving mice were sacrificed. The animals were examined at necropsy, and the pathologic changes in the lungs were evaluated numerically as scores based on 100 as the maximal degree of tuberculous involvement.

Evaluation of results: The criteria used for the evaluation of the effectiveness of a given medication regimen upon experimental tuberculous infection of mice were the median survival time, the survival ratio of treated and untreated animals, and the average pathology ratio. The latter was obtained by dividing the average pulmonary pathology score of the untreated controls by that of the treated group.

Observations

Antituberculous Effectiveness in Mice

The therapeutic effects of the thiohydantoin derivative (Win 5211) on the course of infection due to the isoniazid- and streptomycin-susceptible H37Rv strain of *M. tuberculosis*, the isoniazid-resistant and the streptomycin-resistant variants of H37Rv, and the Ravenel (bovine) strain are demonstrated by the data presented in table 1.

It may be seen that the thiohydantoin derivative showed significant therapeutic activity against experimental disease of mice induced by *M. tuberculosis*, strain H37Rv, streptomycin-susceptible, and a highly resistant variant strain thereof.

The compound also exhibited suppression of isoniazid-resistant tuberculosis of mice. Due to the low mortality in the untreated controls, it was necessary to evaluate the results of this experiment by means of microscopic examination of tissues. The findings showed pronounced suppression of acid-fast bacilli in the lungs of mice treated with the thiohydantoin compound, while the number of mycobacteria in the lungs of mice treated with isoniazid was approximately the same as in the controls which received no medication.

The thiohydantoin derivative (Win 5211) failed to exhibit any activity against murine tuberculosis induced by a bovine strain of *M. tuberculosis*.

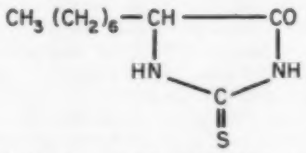

FIG. 1. Chemical structure of 5-heptyl-2-thiohydantoin (Win 5211).

TABLE 1

THE EFFECT OF A THIOHYDANTOIN DERIVATIVE (WIN 5211) ON EXPERIMENTAL TUBERCULOSIS OF MICE

Strain of *M. tuberculosis*	Drug	Dose	Median Survival Time	Number of Survivors / Number of Inoculated	Average Pathology Score	Average Pathology Ratio*
		mg/kg/day	*days*			
Human H37Rv	Thiohydantoin	150	>31	15/18	63.5	1.44
	Thiohydantoin	300	>31	17/17	35.5	2.58
	Thiohydantoin	600	>31	18/18	23.0	3.99
	Untreated	—	19.5	5/20	91.5	—
Human H37Rv, streptomycin-resistant	Thiohydantoin	150	>31	7/10	73.0	1.22
	Thiohydantoin	300	>31	9/10	44.0	2.02
	Thiohydantoin	600	>31	9/9	21.0	4.25
	Dihydrostreptomycin	0.5†	13	1/10	100.0	0.89
	Dihydrostreptomycin	5.0†	13.5	1/10	100.0	0.89
	Untreated	—	13	3/19	89.4	—
Human H37Rv, isoniazid-resistant	Thiohydantoin	300	>31	9/10	23.5	1.19
	Thiohydantoin	600	>31	8/10	15.0	1.87
	Isoniazid	5	>31	9/10	30.9	0.91
	Isoniazid	50	>31	8/10	34.5	0.81
	Untreated	—	>31	16/20	28.0	—
Bovine, Ravenel	Thiohydantoin	150	15.5	0/10	96.6	1.03
	Thiohydantoin	300	16.5	0/10	93.2	1.07
	Thiohydantoin	600	17.0	0/9	77.7	1.29
	Isoniazid	6	>31	10/10	32.7	3.06
	Untreated	—	14.0	0/20	100.0	—

* Untreated/treated.

† Dose per mouse, administered subcutaneously.

Effectiveness in Experimental Tuberculosis of Hamsters

The golden Syrian hamster (*Cricetus auratus*) has been found to be a useful experimental animal for the evaluation of antituberculous drugs (4). Untreated tuberculosis in the hamster is invariably fatal, but the disease develops very slowly without the acquisition of tuberculous hypersensitivity and with very little tendency to caseation. As in man, the infection has a marked predilection for the lungs, irrespective of the route of inoculation.

A total of 110 hamsters weighing 60 to 80 gm. were infected by subcutaneous injection of 0.1 mg. (moist weight) of *M. tuberculosis* H37Rv into the left groin. The experiment was designed in such a way that treatment of one group of 40 animals was started on the third day, while treatment of the second group of 40 hamsters was delayed until the twenty-first day of infection. The remaining 30 animals served as infection controls, 10 of which were sacrificed twenty-one days after infection to demonstrate that the infection had been uniformly established.

The thiohydantoin derivative (Win 5211) was administered to the hamsters as a powder

mixed with the ground feed at concentrations of 0.1, 0.2 and 0.4 per cent. Para-aminosalicylic acid (PAS) was administered as a reference drug at a concentration of 0.4 per cent. The nontreated animals were fed plain ground diet and were kept under the same conditions as the medicated animals. Individual weights of the animals were recorded once a week. Animals which died during the course of the experiment were examined and records were made of the extent of the disease seen at necropsy.

The experiment was terminated one hundred and twenty-five days after infection, when half of the infected untreated animals had died. All survivors were sacrificed at that time, and the extent of the tuberculous involvement of the appropriate organs was recorded for each animal. The gross tuberculous lesions in the lungs, spleen, liver, lymph nodes, and at the site of infection were tabulated according to a scoring system, the maximal total score per hamster being 100. From this the average pathology score and the pathology ratio (untreated/treated) were calculated. In addition, tissues from the appropriate organs (lungs, spleen, liver, lymph nodes) were examined histologically to determine the effect of medication on the progress of the disease in the tissues.

The thiohydantoin compound (Win 5211) exerted appreciable suppressive and therapeutic effect on experimental tuberculosis in the hamster (table 2). When medication was instituted on the third day of infection, 0.1 per cent of the drug showed slight suppressive activity, comparable with that of 0.4 per cent of PAS under identical conditions. Concentrations of 0.2 and 0.4 per cent in the diet exerted proportionately favorable effects on survival, pathology, and body weight of the animals. Microscopically, the lesions in the predilectory organs were rare, showing regression, healing, and significant reduction or complete clearance from tubercle bacilli.

When medication was delayed until twenty-one days after infection, 0.1 per cent of the thiohydantoin compound exerted slight, and 0.2 per cent moderate, antituberculous activity. The concentration of 0.4 per cent of the drug proved to be highly effective by prolonging the life of the animals, by suppressing and

TABLE 2

THERAPEUTIC EFFECT OF A THIOHYDANTOIN DERIVATIVE (WIN 5211) ON EXPERIMENTAL TUBERCULOSIS OF HAMSTERS (STRAIN H37Rv)

Compound	Start of Medication	Total Days Treated	Dose in Diet	Survivors		Average Pathology		Per Cent of Weight Gain
				Number	Per Cent	Score	Ratio	
	days after infection		*per cent*					
Untreated controls	—	—	—	10/20*	50	80.8	—	24.4
Thiohydantoin	3	122	0.1	8/10	80	51.7	1.56	23.5
Thiohydantoin	3	122	0.2	7/10	70	9.3	8.69	65.7
Thiohydantoin	3	122	0.4	10/10	100	6.3	12.80	52.6
Thiohydantoin	21	104	0.1	7/10	70	48.7	1.66	47.1
Thiohydantoin	21	104	0.2	10/10	100	24.0	3.37	43.3
Thiohydantoin	21	104	0.4	9/10	90	4.5	17.90	43.1
PAS	3	122	0.4	8/10	80	49.8	1.62	39.4
PAS	21	104	0.4	8/8	100	51.6	1.56	28.7

* The experiment was terminated when 50 per cent of the infection controls succumbed to infection (125th day).

reducing tuberculous lesions in the organs of predilection, and by showing no harmful influence on the weight of the animals.

All medication regimens were well tolerated, and no side effects attributable to the drug were observed in any of the animals. On the basis of this experiment, it appears that 5-heptyl-2-thiohydantoin is a potent antituberculous drug and significantly more effective than PAS.

Effect of the Thiohydantoin Derivative in Multiple-Drug Therapy in Mice

Multiple-drug therapy with two or more chemotherapeutic agents is an established and useful practice in many diseases, particularly tuberculosis. In order to investigate the effectiveness of the thiohydantoin derivative (Win 5211) as a supplementary drug to established antituberculous drugs, an experiment was carried out to study the effect of such therapy upon mice infected with tubercle bacilli of human origin. The drug regimens studied were the thiohydantoin derivative and dihydrostreptomycin and thiohydantoin and isoniazid.

Seventeen groups of 10 Swiss mice each were inoculated intravenously with 0.2 mg. (moist weight) of virulent human tubercle bacilli, strain H37Rv. Fifteen groups were treated for a total of thirty-five days, starting on the third day after infection; two groups were left untreated and served as infection controls. An additional group of noninfected mice was included to serve as drug controls.

The thiohydantoin derivative (Win 5211) was administered as a suspension in gelatin, at dosage levels of 100, 200, and 400 mg. per kg. per day, by stomach tube, twice daily, five days a week, for thirty-five days. Isoniazid was mixed with the ground feed at a concentration of 0.0015 per cent and supplied *ad libitum*. Dihydrostreptomycin was administered at a subeffective dosage level of 250 γ per mouse, once daily, subcutaneously, for five days a week for a total of thirty-five days. The infection controls received the same volume of gelatin solution by gavage twice daily for the same period of time. The animals were observed for a total of thirty-eight days, weighed once a week, and checked daily throughout the experiment. Mice which died during the experiment were examined at autopsy, and a record was kept of the extent of tuberculous lesions in the lungs. At the end of the test all surviving animals were sacrificed and examined at necropsy.

Treatment with Thiohydantoin and Dihydrostreptomycin

The first part of this study, in which graded doses of the thiohydantoin derivative were administered simultaneously with a subeffective dose of dihydrostreptomycin, is summarized in table 3.

From the results recorded in table 3, it may be observed that concurrent administration of the thiohydantoin and dihydrostreptomycin resulted in an increased antituberculous effect. All mice that received the thiohydantoin compound at 200 and 400 mg. per kg. alone or along with dihydrostreptomycin survived. The maximal suppression of pathologic changes was noted in the group which was medicated with 400 mg. per kg. of thiohydantoin along with dihydrostreptomycin. Mice that received 200 mg. per kg. of the compound and dihydrostreptomycin showed slightly more pathologic changes in the lungs than those receiving the same dose of the thiohydantoin derivative alone, but the difference is considered to be insignificant.

TABLE 3

EFFECT OF MULTIPLE-DRUG THERAPY WITH THIOHYDANTOIN DERIVATIVE (WIN 5211) AND DIHYDROSTREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS (H37Rv IN MICE)

Medication		Mice per Group	Median Survival Time	Results			
				Survivors		Average Pathology	
Thiohydantoin*	Dihydrostreptomycin†			Number	Per Cent	Score	Ratio
mg/kg/day	*γ/day*		*days*				
100	—	10	>38	6/10	60	86.8	1.03
100	250	10	>38	7/10	70	72.9	1.23
200	—	10	>38	10/10	100	40.8	2.20
200	250	10	>38	10/10	100	44.2	2.03
400	—	8	>38	8/8	100	24.0	3.74
400	250	10	>38	10/10	100	15.3	5.86
—	250	10	>38	6/10	60	86.5	1.04
Untreated controls		20	20.5	0/20	0	89.8	—

* Administered by gavage.

† Administered by subcutaneous injection, 0.2 ml. per mouse.

Treatment with Thiohydantoin and Isoniazid

In the second part of this study, mice were medicated concurrently with graded oral doses of the thiohydantoin along with 0.0015 per cent of isoniazid administered in the diet. The effects of these regimens upon the tuberculous process in the mice are presented in figure 2.

It may be seen that in every instance the two-drug therapy proved to be superior to therapy with either drug given alone at the dosage levels used. The maximal effect was obtained when 400 mg. per kg. of the thiohydantoin were administered with isoniazid. This regimen resulted in 100 per cent survival of the animals for the duration of the experiment, and an average pathology score of 7.5, compared with pathology scores of 32.7 for isoniazid alone and 24.0 for 400 mg. per kg. of the thiohydantoin given alone. The few tuberculous lesions which were found in the lungs of these animals had the appearance of minute, nonprogressive cicatrized lesions and, microscopically, only very small numbers of acid-fast bacilli could be found. A distinct increase of antituberculous activity was noticed by increasing the dose of the thiohydantoin derivative.

DISCUSSION

It has been demonstrated in these experiments that 5-heptyl-2-thiohydantoin (Win 5211) produced beneficial effects in severe experimental tuberculosis in the mouse and in the hamster. The compound was found to be highly active in suppressing and retarding the progress of the experimental disease without producing visible toxic reactions. Extensive tolerance and toxicity studies carried out in these laboratories have shown that the thiohydantoin (Win 5211) possesses a low degree of toxicity and is well tolerated by mice, dogs, and monkeys. The acute oral ALD_{50} in mice was found to be more than 10 gm. per kg. Oral admin-

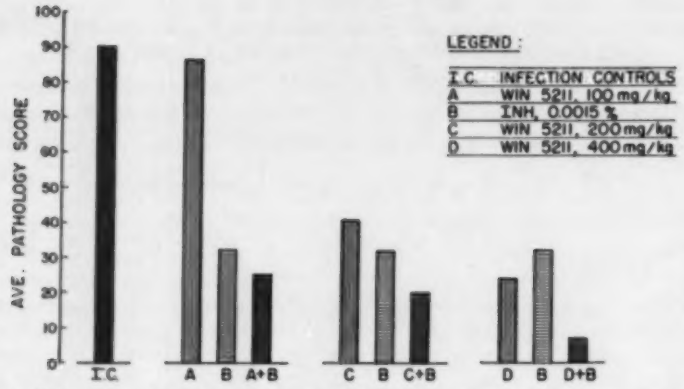

FIG. 2. Effect of multiple-drug therapy with Win 5211 and isoniazid on experimental tuberculosis of mice.

istration of this compound to dogs for periods of three weeks and fourteen weeks was well tolerated at daily doses up to 100 mg. per kg. Equally good tolerance was observed in rhesus monkeys which received the compound for three and fourteen weeks at daily doses up to 160 mg. per kg. The only significant deviations observed upon prolonged administration of the drug were mild hyperplastic changes of the thyroid epithelium. These changes were not reflected in the general behavior or appearance of the animals and disappeared soon after the medication was discontinued.

Similar changes of the thyroid glands were observed in tuberculous patients after prolonged administration of a derivative of diaminodiphenylsulfone (Promizole®) (5). Clausen and Kjerulf-Jensen (6), and Komrower (7), reported in 1951 that prolonged treatment with PAS caused myxedema and goiter of the thiouracil type in some patients which disappeared after discontinuance of PAS medication. Torikai and Kumaoka (8) recently reported that PAS inhibits the binding of tyrosine and elemental iodine, thus exerting a mild antithyroid effect. The development of severe myxedema and enlargement of the thyroid gland is a rare side effect in tuberculous chemotherapy, but a mild antithyroid effect may not be an undesirable property for an agent of this type.

Following the termination of laboratory "screening" studies, the thiohydantoin derivative (Win 5211) was given a limited trial in tuberculous patients by other investigators. Their results showed that the response to this drug alone was not sufficient to justify its use as an alternative to isoniazid or dihydrostreptomycin (9). The present laboratory studies suggest, however, that it may offer a desirable alternative to PAS for combination therapy.

SUMMARY

The compound 5-heptyl-2-thiohydantoin (Win 5211) is a new antituberculous agent of a different chemical group than known antituberculous drugs. The compound was found to be effective in the treatment of experimental tubercu-

losis of mice and hamsters infected with human tubercle bacilli. Similar results were attained when a strain highly resistant to streptomycin was used to infect the animals. The compound was active against tuberculosis induced in mice by isoniazid-resistant mycobacteria, but failed to show any beneficial results in bovine tuberculosis in the same species. Experiments in hamsters infected with human tubercle bacilli indicate that this thiohydantoin derivative is significantly more effective than PAS, and the evidence suggests that it could be used effectively for multiple-drug therapy at lower daily doses than PAS.

Concurrent administration of the thiohydantoin derivative and dihydrostreptomycin, as well as the thiohydantoin and isoniazid, suppressed tuberculous infection in mice considerably more effectively than medication with each of the drugs separately.

The thiohydantoin compound was found to be well tolerated by laboratory rodents, dogs, and monkeys, the only side effects observed being mild reversible hyperplasia of the thyroid glands, which was observed upon prolonged medication of dogs and monkeys. Tolerance to the thiohydantoin was superior to that to PAS.

Incomplete clinical studies indicate that the possible field of usefulness of this compound might be as an adjuvant to other chemotherapeutic agents, in much the same manner as the use of PAS.

Sumario

El Efecto de la 5-Heptil-2-Tiohidantoína sobre la Tuberculosis Experimental

La 5-heptil-2-tiohidantoína (Win 5211) es un nuevo producto tuberculostático que pertenece a un grupo químico distinto del de las drogas antituberculosas conocidas. El compuesto resultó eficaz en el tratamiento de la tuberculosis experimental de los ratones y los *Cricetus* infectados con bacilos tuberculosos humanos. Se obtuvieron resultados semejantes al usar una cepa sumamente resistente a la estreptomicina para infectar a los animales. El compuesto se mostró activo contra la tuberculosis inducida en los ratones por micobacterias isoniacidorresistentes, pero no reveló el menor efecto beneficioso en la tuberculosis bovina en la misma especie. Los experimentos en los *Cricetus* infectados con bacilos tuberculosos humanos indican que el Win 5211 es decididamente más eficaz que el PAS y los datos disponibles indican que podría usarse eficazmente para terapéutica combinada a dosis diarias más bajas que las de PAS.

La administración concurrente de Win 5211 y de dihidroestreptomicina, así como de Win 5211 e isoniacida, suprimió la infección tuberculosa en los ratones en forma considerablemente más eficaz que la medicación con cualquiera de estas drogas por separado.

El Win 5211 se mostró bien tolerado por los roedores de laboratorio, perros y monos, consistiendo los únicos efectos colaterales observados en hiperplasia reversible del tiroides, que se observó tras la medicación prolongada de perros y monos. La tolerancia al Win 5211 fué superior a la ofrecida al PAS.

Incompletos estudios clínicos indican que la posible esfera de utilidad de este compuesto tal vez sea como coadyuvante de otros productos quimioterapéuticos, en forma muy semejante al uso del PAS.

Resume

L'effet de 5-Heptyl-2-Thiohydantoine sur la tuberculose expérimentale

Le 5-heptyl-2-thiohydantoine (Win 5211) est un nouvel agent tuberculostatique appartenant à un groupe chimique différent de celui des drogues antituberculeuses connues. Ce composé a été trouvé efficace dans le traitement de la tuberculose expérimentale de la

souris et du hamster innoculés avec le bacille tuberculeux humain. Des résultats analogues ont été obtenus lorsque l'animal était inoculé avec une souche très résistante à la streptomycine. Le composé a démontré son activité contre la tuberculose des souris inoculées avec des mycobactéries isoniazido-résistantes, mais, dans ces mêmes espèces animales, les résultats n'ont pas été satisfaisants dans la tuberculose d'origine bovine. L'expérimentation sur les hamsters inoculés avec les bacilles tuberculeux humains indique que Win 5211 est significativement plus efficace que le PAS; et les données obtenues suggèrent qu'à doses inférieures à celles du PAS il pourrait être efficacement utilisé dans la thérapeutique combinée.

L'administration simultanée de Win 5211 et de dihydrostreptomycine, de même que celle de Win 5211 et d'isoniazide, a stabilisé l'infection tuberculeuse de la souris de façon plus effective que l'administration séparée de chacune de ces drogues.

Le Win 5211 a été bien toléré chez les rongeurs, les chiens et les singes de laboratoire, la seule réaction observée était une légère hyperplasie réversible de la thyroïde qui se manifestait chez les chiens et les singes quand la médication était prolongée. La tolérance à Win 5211 était supérieure à celle enregistrée pour le PAS.

Des études cliniques incomplètes indiquent que le champ d'application possible de ce composé pourrait être celui de médication adjuvante aux autres agents chimiothérapeutiques, sensiblement comme le PAS.

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THE TITRATION OF CORD FORMATION AS RELATED TO VIRULENCE OF MYCOBACTERIA[1,2]

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INTRODUCTION

Investigators are constantly seeking new insight into the chemical and physiologic complexities of tubercle bacilli, and they are particularly concerned with uncovering the factor or factors responsible for the virulence of the organisms. The search continues for various complex mechanisms responsible for the disease-producing capacity of one microbial strain while another, identical in appearance, is incapable of producing disease; and still another, also with identical cultural characteristics, provides a vaccine. Since the recognition of avirulent and attenuated variants of the virulent varieties of *Mycobacterium tuberculosis*, this has been a perplexing problem, and any property which indicates a distinguishing feature among the three merits further investigation.

In the last decade attention was focused on one such property by Middlebrook, Dubos, and Pierce (1) when they described "serpentine cord formation" in cultures of virulent mammalian tubercle bacilli grown in Tween®-albumin medium. The bacilli lie parallel in compact, ropelike formation, sometimes coiling and having the appearance of serpents and sometimes forming a more blunt "river of logs."

It should be noted that Robert Koch, in his early studies on the tubercle bacillus, observed and depicted a growth which was typical of colonies of virulent tubercle bacilli on coagulated human serum (2). His illustrations, presented in figure 1, bear a remarkable resemblance to those now familiar in current medical literature and represent finesse in observation with the tools available in those days. Interest in this feature, however, remained dormant for many years until Middlebrook's work provoked a functional interpretation of significance which provided the stimulus for more detailed investigations.

Yegian and Kurung (3) noted that human serum or plasma added to the liquid Tween-albumin medium would stimulate this characteristic growth of tubercle

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[2] This study was made possible by grants from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association; and from the North Dakota Tuberculosis and Health Association and from the North Carolina Tuberculosis Associations.

[3] The cord-formation titration aspect of this paper represents a study made in partial fulfillment of the requirements for a graduate degree in the Department of Bacteriology, University of North Carolina, Chapel Hill, North Carolina.

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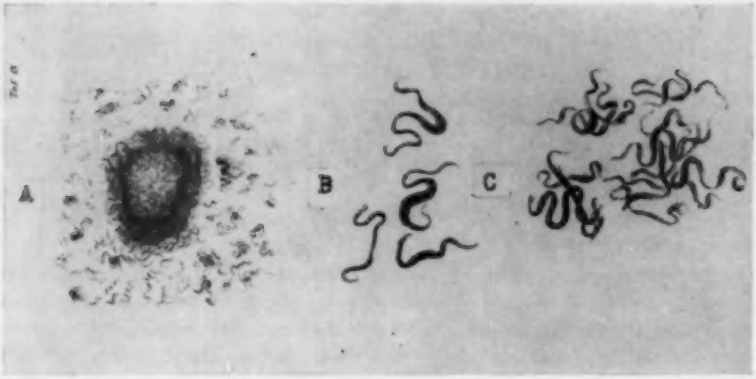

FIG. 1. Reproduced from Koch (2). *A*. An imprint from a culture growing on a solid medium of coagulated human serum. The whorls, coils, and strands were composed of tubercle bacilli generally in parallel. (34 ×) *B*. Higher magnification of strands shown in *A*. Numerous individual bacilli may be seen to lie in the so-called "log jam" arrangement for, as Koch stated: "That these colonies are formed only by tubercle bacilli, one will soon see when they are stained . . . and examined under high magnification." (300 ×) *C*. Tubercle bacilli that have grown in cord formation, taken from culture grown out of a tuberculous focus of a human kidney. The arrangement of the bacilli is characteristic. (300 ×)

bacilli. This laboratory (4, 5), using human plasma in liquid medium containing varying concentrations of Tween 80, found a correlation in the capacity of attenuated strains of tubercle bacilli to maintain cords in increasing concentrations of the Tween with their capacity to produce continuing allergy in guinea pigs.

It was suspected that the intensity of the intracellular binding in cords was related to the virulence or potency of a strain and, therefore, it became necessary to develop a method for quantitatively determining this intensity.

In the present study a technique is described which permits the quantitative measurement of the capacity of avirulent, attenuated, and virulent mycobacteria to form and maintain their own characteristic degree of cordlike growth. The procedure involved two principles: (*1*) the use of human plasma to stabilize and bring more consistency to the process of cord formation; (*2*) the use of Tween 80 in increasingly higher concentrations in an attempt to disperse corded bacilli or prevent formation of cords in liquid medium and, thus, establish an end point of their capacity to maintain cords. The relationship of virulence to this cording phenomenon was also evaluated.

MATERIALS AND METHODS

The basal medium of Dubos (6) was distributed in 25-ml. volumes in Boston round bottles of 500-ml. capacity. Tween 80 (a polyoxyethelene derivative of sorbitan monoöleate) was added to the medium in concentrations ranging from 0.000 to 0.110 per cent at 0.005 per cent intervals. The medium was sterilized at 15 pounds of pressure for fifteen minutes. Bovine albumin, fraction V, which had been sterilized by filtration through a washed Seitz filter, was added aseptically to all bottles in a concentration of 0.5 per cent.

Serum separated from recently out-of-date (twenty-two to twenty-nine days old) human blood obtained from a blood bank was added aseptically in a concentration of 5 per cent. Eleven strains of mycobacteria[5] of human and bovine origin were used in this study and encompassed virulent, attenuated, and avirulent variants. Five human strains included two virulent ones (H37Rv and another recently isolated –before chemotherapy– from a tuberculous patient and termed "wild"), one attenuated (R1Rv), and two avirulent variants (H37Ra and R1Ra). Five bovine strains included one virulent (Ravenel) and four attenuated BCG populations (two from Dr. Rosenthal, one from Dr. Birkhaug, and one from Dr. Aronson). Studies were also made of two smooth, virulent strains (one avian, Sheard, and one bovine, Copenhagen), and several other drug-susceptible and drug-resistant strains. These observations are not included in the graphic presentation.

All cultures were maintained on Löwenstein medium. Prior to their use as inocula in the experiment, they were transferred in Tween-albumin medium three times to obtain maximal dispersed growth. Approximately 0.5 ml. of an eight-day culture grown in Tween-albumin medium was transferred into 25 ml. of the special titration medium. After seven and fourteen days, smears were made by placing 1 drop of the titration culture on a microslide and staining by the Ziehl-Neelsen technique; these were examined microscopically under low-power and oil-immersion magnification. The titer or end point was determined only under low power (100 ×).

Results

The cord-formation titer is interpreted as the highest concentration of Tween 80 at which cording is discernible at low-power magnification, the next highest concentration being above the end point and giving marked dispersion of the cords. These cord-formation titers or end points for several of the strains studied are shown in figure 2, which pictorially demonstrates the appearance of these cords at representative levels of Tween concentrations. A "one-tube" (flask) variation was observed occasionally and is not represented in the figure.

The rough, virulent strains grew as long, compact "ropes" formed by the bacilli lying extremely close together and parallel to each other. The "ropes" were several bacilli wide, sometimes coiling and sometimes having the appearance of loops. Usually the colonies had no central core but were composed entirely of these long, intertwining "wormlike" formations. So tightly did the bacilli adhere together, it was difficult to make out individual cells even under high magnification. This serpentine pattern persisted in Tween 80 concentrations of more than 0.09 per cent.

It was found that there was not a common titer which was acceptable to all

[5] All strains were obtained from the Trudeau Culture Bank through Dr. William Steenken of Trudeau Laboratory, Saranac Lake, New York, with the exception of the "wild" human strain and the BCG strains. BCG-1 and BCG-4 were from the Tice Laboratories through the kindness of Dr. Sol Rosenthal; #1 was received in 1951 and #4 in 1956. BCG-2 was obtained from Dr. Konrad Birkhaug, New York State Department of Health. BCG-3 came from Dr. Joseph Aronson, Henry Phipps Institute.

The numbering system for the BCG strains used in this paper does not correspond with that used in a previous publication (Am. J. M. Sc., 1953, *225*, 410):

#1 in the previous paper is now #3
#2 in the previous paper is now #2
#3 in the previous paper is now #1
#4 is a strain not used previously

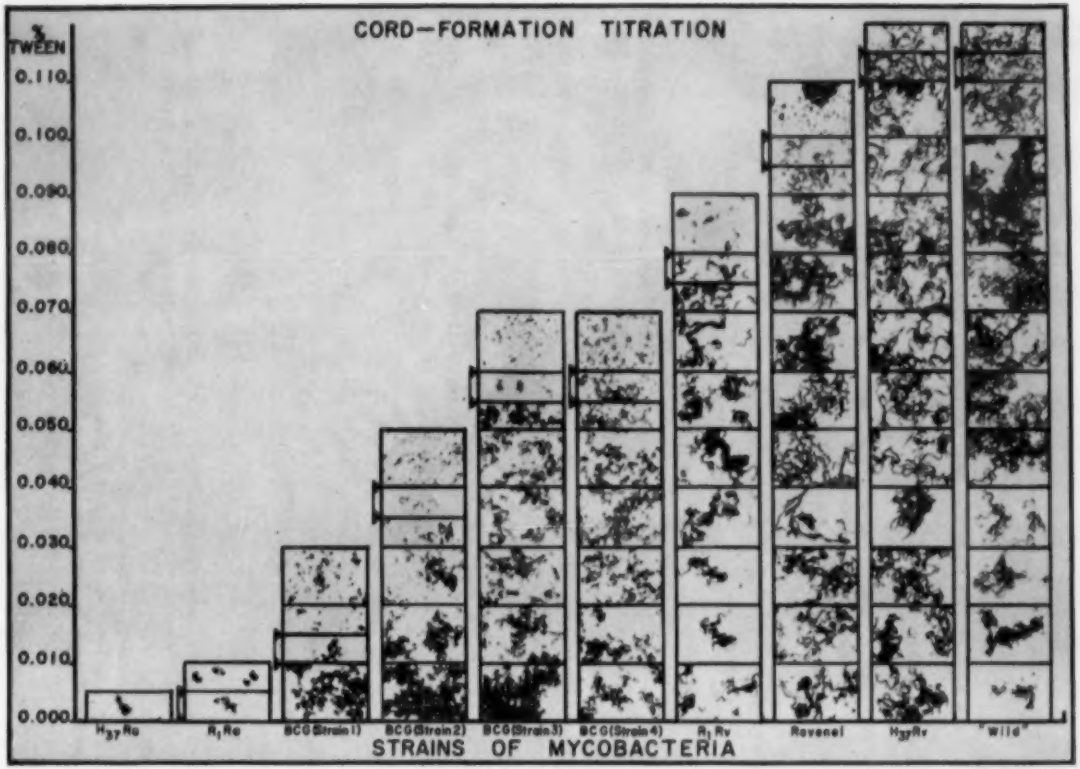

FIG. 2. A graphic pictoral representation of avirulent, attenuated, and virulent strains of mycobacteria demonstrating their specific capacity to maintain cords in increasing concentrations of Tween® 80. The end point or titer of each strain is indicated by ↧. The photomicrograph above the indicated end point illustrates the Tween concentration of significant dispersion. (22 ×)

four of the attenuated BCG strains but, rather, the titer varied for each, depending upon the origin of the strain. The strains of BCG received from various sources (BCG-1, -2, -3, -4) gave specific titers which were consistent but which differed among strains, with the exception of BCG-3 and BCG-4. This consistency was true even after long periods (up to four years) of repeated transplants in this laboratory and also with repeat cultures from the parent laboratory. The strains BCG-1 and BCG-4 were from the same source but were considered different from each other by the parent laboratory. Therefore, the attenuated strains had a cord-formation titer which was specifically their own but varied among strains with the given strain, with the exception of BCG strains 3 and 4, which were equivalent. All titers of these attenuated strains were below a concentration of 0.09 per cent Tween 80.

The cord-formation titers of the attenuated strains, BCG-1, -3 and R1Rv corresponded with duration of allergy in animals after eight months' observation as previously reported (4, 5); i.e., those with the lowest titers gave the shortest duration of allergy, whereas those with higher titers gave a correspondingly higher percentage of animals which reacted to tuberculin for a longer period of time. Subsequently, correlation with duration of allergy has been manifest for BCG strain 2, and there has been agreement between cord-formation titration and volume of allergic response at eight months for BCG strains 1, 2, 3, 4, and R1Rv as described in the footnote.[6]

There is also a correlation between the titer and omental index (7) of a given strain and between the titer of attenuated strains and their immunogenic properties (8) in that those with higher titers confer a higher degree of immunity. Therefore, duration of allergy, volume of allergic response, omental index and cord-formation titer have all demonstrated an equivalence of values for each of the several strains tested which verified the suggested variations in potency.

[6] When equivalent quantities of viable bacilli have been inoculated into groups of guinea pigs, the resultant allergy has varied in its duration, consistent with the variation in cord titer of the bacilli. For instance, in one series of 200 animals, eight months following vaccination it was found that 59 per cent of those inoculated with BCG-strain 1 gave a skin reaction to OT 1:1,000 (approximately equivalent to 0.0002 PPD) in comparison with 81 per cent with BCG-strain 2, 94 per cent with BCG-strain 3, and 100 per cent with R1Rv.

Similar variations have been demonstrated in the volume of allergic response elicited by the various strains. Statistical analysis was made of the volume of skin reactions eight months after vaccination by testing with OT 1:1,000. These reactions were measured with calipers and calculated as the volume of a segment of a sphere: $V = \frac{1}{6}\pi h\,(h^2 + 3r^2)$. In this series, the mean volumes were: BCG-strain 1—56 cu. mm., BCG-strain 2—81 cu. mm., BCG-strain 3—99 cu. mm., BCG-strain 4—100 cu. mm., and R1Rv—132 cu. mm. Comparisons, based on students' ratio $\left(t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(S\bar{x}_1)^2 + (S\bar{x}_2)^2}}\right)$, were made of these differences between the strains. Comparison between BCG-strain 1 and BCG-strain 2 gave t value of 2.1; between BCG-strain 2 and BCG-strain 3, gave t value of 2.4; between BCG-strain 3 and BCG-strain 4, gave t value 0.2; and between BCG-strain 4 and R1Rv gave t value of 3.1. The differences in volume between the groups were statistically significant in each instance except between BCG-strain 3 and BCG-strain 4, when the difference was slight and insignificant.

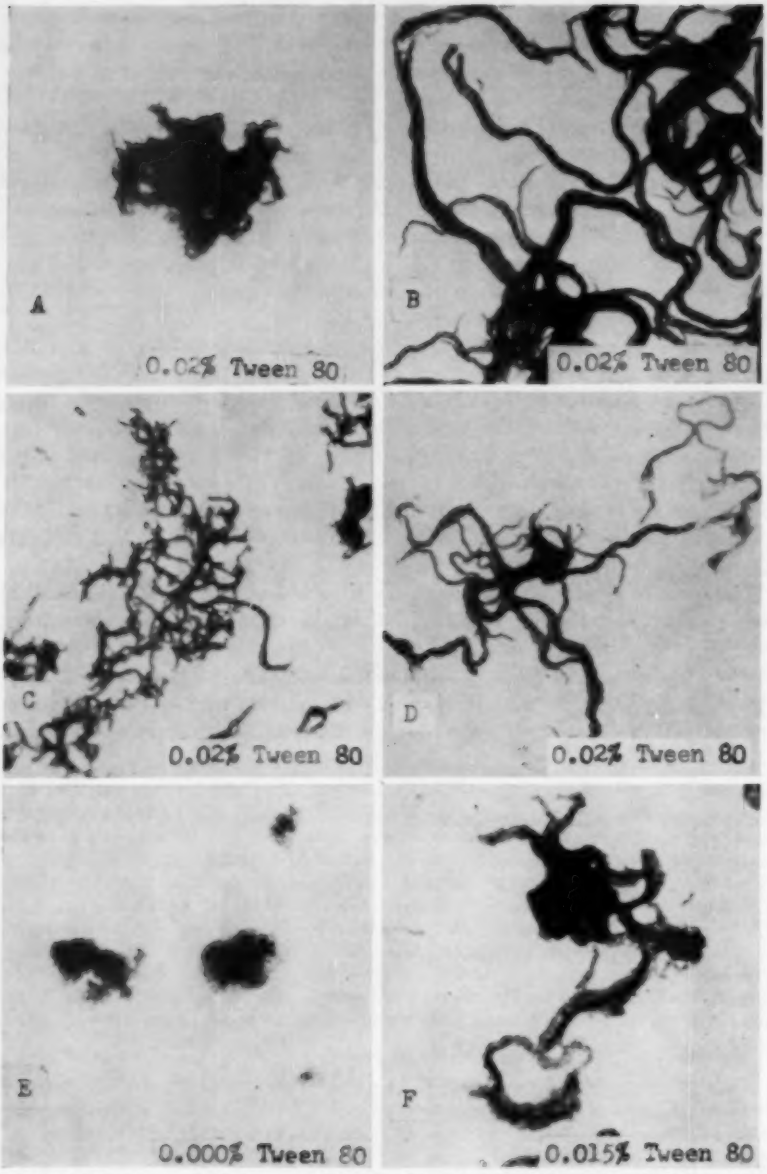

FIG. 3. Qualitative variation in cords of several strains of mycobacteria: (*A*) The rough, avirulent, H37Ra; (*B*) A rough, virulent, H37Rv; (*C*) An attenuated strain, BCG-3. (*D*) The attenuated R1Rv, and two smooth, virulent strains, the avian (Sheard (E)) and the bovine (Copenhagen (F)). (380 ×)

For example, R1Rv gave the highest cord-formation titer and also gave the highest response in animals, as determined by the value of omental index, duration of allergy, and volume of allergic response. The pattern of cording of attenuated strains was microscopically similar to that of the virulent strains except that the cords appeared shorter and the cells were held more loosely within the coils and often originated from a central core in the colonies.

The avirulent strains did not form cords. In lower concentrations of Tween 80, only clumps of avirulent bacilli could be detected while, at higher concentrations, isolated cells and smaller clumps were seen on the slides.

Individual differences in quality of cord formation, such as compactness of the cords, length of cords, and the presence of central core in the colonies, may be seen in the accompanying photomicrographs (figure 3), all taken at a low Tween 80 concentration (0.02 per cent).

It must be stated that not all virulent strains present the phenomenon of cord formation as might be expected, because two smooth, virulent strains (the avian, Sheard, and the bovine, Copenhagen) failed to respond in the manner of the rough, virulent strains discussed above. The avian strain, although virulent for chickens at the time of the study, did not form cords at any concentration of Tween 80. Indeed, it failed to grow in cord arrangement even in the absence of Tween 80, as shown in figure 3.

The Copenhagen strain (a smooth, dysgonic strain) did form cords in low Tween concentrations (0.00–0.02 per cent), but these were of different character (figure 3) than the typical, tightly knit, compact, long ropelike structures formed by rough strains such as Ravenel, H37Rv, or the average strain recovered from a patient. The Copenhagen cords were maintained at Tween 80 concentrations of 0.06 per cent if examined at seven days; but after fourteen days dispersion had occurred to such an extent that cording was not observed in concentrations of Tween 80 of more than 0.02 per cent. This change or reduction in cord titer after fourteen days was not observed with any mycobacteria other than the smooth, virulent, bovine strain. Even with this low, fluctuating titer, this strain was pathogenic for rabbits, producing rapidly progressive disease which was fatal within three to five weeks when inocula of 0.1 mg. were used.

Discussion

It has been established that rough, eugonic, mammalian strains of tubercle bacilli form cords in culture. This fact holds true for recently isolated strains from sputum and resected lung tissue as well as for stock laboratory strains such as H37Rv. Such strains produce cords which will persist in the presence of the dispersing agent, Tween 80, in concentrations of 0.09 per cent.

Attenuated strains such as BCG and R1Rv, also form cords, but these are broken up, and the bacilli are dispersed in the presence of lower Tween concentrations than those required for breaking up the virulent strains. Furthermore, the lower the cord formation titer, the shorter is the duration of allergy and less intense is the volume of allergic response produced in guinea pigs. The cord-formation titers of the BCG cultures from the different laboratories are not the same (except possibly for BCG strains 3 and 4). This indicates there have been in use

several distinct and separate BCG strains, each with a different potency, originating from separate laboratories.

It must be pointed out that only one of the virulent mammalian strains used in this study failed to produce a cord titer corresponding to the animal response ordinarily evoked by it. The cord titer of the Copenhagen strain—a smooth, dysgonic, bovine strain—was far less than 0.09 per cent Tween 80 although this strain is capable of producing progressive disease in rabbits. No mention has been made in the literature of cord formation of smooth strains of tubercle bacilli, and it may well be that the factor (or factors) responsible for the smoothness of a strain in some way interferes with the capacity of the tubercle bacilli to form cords.

No evidence has been brought forth as yet that avian strains, even virulent ones, will form cords. Knaysi and associates (9), in studying the cytology of the Sheard strain with electron and light microscopy, have observed in submerged cultures the presence of a "slime" layer composed chiefly of volutin. Possibly this "slime" layer masks a component responsible for cord formation, which Noll and Bloch (10) have termed "cord factor," and prevents the culture of avian bacillus from clinging together in the characteristic cord formation.

It should be noted that a "one-tube" variation in end points either up or down may occur occasionally with any of these strains without cause for concern over the stability of the quantitative potency of a culture.

It is significant to note that the cord-formation titer of a given strain can be altered, depending upon the method of maintaining the strain in the laboratory. Prolonged and continuing transfers in liquid Tween-albumin (bovine fraction V) medium have caused a decrease in the titer of the R1Rv strain with a corresponding drop in volume and duration of allergic response in guinea pigs as well as a decrease in omental index, whereas the converse is true with BCG strains (11). Strains carried on Löwenstein medium have been found to maintain a relatively stable cord-formation titer. This is the basis for maintenance of the strains on this solid medium, with three transfers to liquid medium immediately before a titration is set up.

In order to obtain standard titrations, particular attention must be given to details in setting up the test. The surface area of the container used has been shown to affect the end points. If the titrations are carried out in test tubes or vials containing 5 ml. of medium rather than the 500-ml. Boston round bottles containing 25 ml. of medium, the end point tends to fall approximately two dilutions below that established originally in this laboratory for a given strain. Because of the space required for incubation and the large volume of medium needed for the 500-ml. bottles, the cord-formation titration is used for experimental work but not on a routine basis, although cord-formation titration is used routinely as a part of the evaluation of "atypical" mycobacteria. It has become an aid in the determination of potency of the attenuated strains used in preparing vaccines for experimental animals in this laboratory and has been incorporated into the potency standardization of these vaccines.

Thus, for satisfactory results in comparing the cord-formation titration of various strains, a standard method of setting up the test must be chosen and adhered to closely.

Summary

A description is given of cord-formation titration of various strains of tubercle bacilli which can be effected by culturing the bacilli in liquid albumin medium containing 5 per cent human plasma and varying concentrations of Tween® 80. Titration of the capacity of various strains to maintain cords in the presence of these increasing concentrations of Tween is a practicable procedure. This cord-formation titration is consistent with virulence, avirulence, or degree of attenuation of rough, eugonic, mammalian strains.

Two smooth strains, one bovine (Copenhagen) and one avian (Sheard), failed to produce titers equivalent to the rough, eugonic virulent strains or equivalent to their potency as determined by animal inoculation.

Sumario

La Titulación Cuantitativa de la Cordogenia en Relación con la Virulencia de las Micobacterias

Ofrécese una reseña de la titulación de la formación de cordones por varias cepas de bacilos tuberculosos, lo cual puede efectuarse cultivando los bacilos en medio de albúmina líquido que contenga 5 por ciento de plasma humano y concentraciones variables del Tween® 80. La titulación de la capacidad de varias cepas para mantener cordones en presencia de dichas concentraciones crecientes de Tween es un procedimiento práctico. Esta titulación de la cordogenia no se ve afectada por la virulencia, avirulencia o grado de atenuación de las cepas ásperas y eugónicas de mamífero.

Dos cepas lisas, una bovina (Copenhagen) y una aviaria (Sheard), no produjeron títulos equivalentes a los de las cepas virulentas y eugónicas ásperas o equivalentes a éstas en potencia, según se determinó por la inoculación en animales.

Resume

Determination quantitative de la formation de cordes en rapport avec la virulence des mycobactéries

Les auteurs décrivent le titrage de la formation de cordes dans diverses souches de bacilles tuberculeux, qui peut être réalisé par une culture du bacille dans un milieu liquide à l'albumine renfermant 5% de plasma humain et diverses concentrations de Tween® 80. La détermination de la capacité des diverses espèces à maintenir ces cordes en présence de ces concentrations croissantes de Tween est un procédé pratique. La formation de cordes correspond au degré le titre de virulence, d'avirulence ou d'atténuation des souches rugueuses, eugoniques de mammifères. Deux souches lisses, une bovine (Copenhague) et une aviaire (Sheard), n'ont pas produit de titre équivalant à ceux des souches rugueuses eugoniques, virulentes ou équivalentes à leur activité, telle qu'elle est déterminée par l'inoculation à l'animal.

Acknowledgment

The cooperative aid and advice of Dr. D. A. MacPherson, Department of Bacteriology, University of North Carolina, in the capacity of graduate school adviser, and of Dr. H. S. Willis, Superintendent-Medical Director, North Carolina Sanatorium System, is acknowledged with greatest appreciation.

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A NEW TUBERCULOSTATIC PROTEIN ISOLATED FROM BOVINE SPLEEN[1, 2]

QUENTIN N. MYRVIK AND EVA SOTO-FIGUEROA

(Received for publication October 7, 1957)

INTRODUCTION

The marked resistance which some mammalian tissues demonstrate against *in vivo* multiplication of tubercle bacilli suggests that biochemical components associated with certain tissues may play an important role in regulating the multiplication of tubercle bacilli in tuberculous lesions. In this regard, Chahovitch, Vichnjitch, Frajnd, and Schvartz (1) reported that an extract of pepsin-hydrolyzed tuberculous lungs had a tuberculostatic effect. Hirschberg and Arnold (2) examined tissue juice from fresh normal human organs and observed a growth-inhibitive effect on tubercle bacilli with an apparent correlation with the resistance of the organs to tuberculosis. Sarnowiec (3) reported that extracts of the livers, kidneys, or spleens from either healthy or tuberculous guinea pigs possessed little or no tuberculo-inhibitory activity. However, he did observe some activity in liver and kidney extracts provided autolysis was allowed to take place before the preparations were tested.

Nutini and Kreke (4) reported that splenic extracts exerted a toxic effect on hemolytic streptococci and on tubercle bacilli. Corper (5) reported that boiled dog spleen preparations tested *in vitro* possessed tuberculostatic activity. Dubos and Hirsch (6) described a basic peptide preparation derived from calf thymus and also present in bovine spleen. The thymus factor consisted of a basic peptide or a mixture of basic peptides that inhibited the growth of tubercle bacilli under certain conditions. A basic protein has been isolated from bovine spleen by Bloom and Prigmore (7) which is active against *Bacillus subtilis*. The basic polypeptide isolated by Dubos and Hirsch may represent a derivative of the type of basic protein isolated by Bloom and Prigmore. These two classes of basic components isolated from tissue are probably nuclear in origin (8) and may not exist as free circulating components.

The present report describes the isolation and characterization of a tuberculostatic protein in bovine spleen which has not previously been described.

MATERIALS AND METHODS

Microorganisms: The H37Rv, Erdman (human), 4228-4 (bovine), and Ravenel strains of *Mycobacterium tuberculosis* were kindly provided by Dr. William Steenken, Jr., Trudeau

[1] From the Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia.

[2] This study was aided, in part, by grants from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association, made possible by a special bequest from the Estate of Grace Velie Harris; from the Virginia Tuberculosis Association; and a research grant (E-1238) from the Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service.

Sanatorium, Saranac Lake, N. Y. The BCG strain of *Mycobacterium tuberculosis* was obtained from Dr. Russell S. Weiser, Department of Microbiology, University of Washington School of Medicine, Seattle, Washington. The strains of *Mycobacterium smegmatis* and *Mycobacterium phlei* employed were obtained from the stock culture collection in this institution.

Preparation of inocula: The mycobacteria were grown on Youman's modification (9) of Proskauer and Beck's medium at pH 6.8 for six to eight days at 37° C. The surface growth was ground in a sterile mortar, and the homogenized cell mass was suspended in sterile physiologic saline containing 0.04 per cent Tween® 80. The suspension was centrifuged lightly to remove large clumps of cells, and the supernatant fluid was diluted with saline to give approximately 100 "nephelos" units in a Coleman model 9 nephelometer.

Assay method of tuberculostatic activity: Tuberculostatic activity was quantitated by double diluting the components to be tested in a medium (pH 7.0) containing: KH_2PO_4, 0.05 per cent; Na_2HPO_4, 0.12 per cent; asparagin, 0.1 per cent; casein hydrolysate, 0.05 per cent; Ferric ammonium citrate, 0.002 per cent; $MgSO_4$, 0.0005 per cent; glycerin, 0.5 per cent; Tween 80, 0.04 per cent; and Fraction V bovine albumin, 0.25 per cent. Assays were also conducted in the above medium from which Tween 80 was omitted in specified instances. The tubes were inoculated with one drop (0.02 ml.) of the standardized suspension of the mycobacteria and incubated at 37° C. for four to ten days, depending on the species of mycobacteria employed. The end point was the least amount of substance which produced complete inhibition of the test organism as determined by visual inspection.

Analytic procedures: Nitrogen analyses employed the micro-Kjeldahl method outlined in Kabat and Mayer (10). Desoxyribonucleic acid was determined by Seibert's modification of the method of Dische (11). Total carbohydrate was determined by Koehler's modification of the anthrone method (12), based on glucose as a standard. Total phosphorus was determined by the method of Fiske and Subbarow (13).

Paper electrophoresis: Electrophoretic studies were performed on a Precision ionograph at 22° to 25° C. employing veronal buffer, pH 8.6, with an ionic strength of 0.05. Whatman 3MM paper strips were used either in one-half-inch or one-inch widths. Upon completion of a run, the paper strips were dried at 110° C. for thirty minutes and subsequently stained in 0.05 per cent bromphenol blue in 2 per cent acetic acid saturated with $HgCl_2$. The stained strips were washed in several changes of 0.05 per cent acetic acid. A final wash was made with 0.05 per cent acetic acid in 0.05 per cent sodium acetate, after which the strips were dried in an electric oven at 80° C. for thirty minutes.

Paper chromatography: The purified protein preparation was hydrolyzed in 6 N HCl at 103° C. in an oil bath for eighteen hours, employing a reflux distillation apparatus. The acid was removed by vacuum distillation. Ascending paper chromatograms were made on Whatman No. 1 filter paper formed into cylinders and placed in closed jars. Phenol and butanol-acetic solvents were prepared according to Berry and associates (14). The chromatograms were dried and developed by spraying with 0.25 per cent ninhydrin in water-saturated butanol and subsequently drying at 90° C. for eight minutes.

Results

The tuberculostatic activity of the bovine spleen factor was first observed by testing crude aqueous extracts prepared by grinding fresh bovine spleens and diluting with two volumes of distilled water. This slurry was adjusted to pH 3.0 and allowed to extract overnight at 4° C. The clear amber supernate was removed by centrifugation, adjusted to pH 7.0 with 5 N sodium hydroxide, and sterilized by filtration through a Seitz or Selas filter.

Tests of the tuberculostatic activity of the crude extract indicated (table 1) that bovine spleen extract was more active against the BCG and H37Rv strains of *M. tuberculosis* than against the Ravenel strain of *M. tuberculosis*. In a series

TABLE 1

THE TUBERCULOSTATIC ACTIVITY OF CRUDE AQUEOUS EXTRACT OF BOVINE SPLEEN

Organism	Dilution of Extract*					
	6	12	24	48	96	192
M. tuberculosis BCG	−	−	−	−	+++	++++
M. tuberculosis H37Rv	−	−	−	−	+++	++++
M. tuberculosis Ravenel	−	−	+++	++++	++++	++++
M. smegmatis	++++	++++	++++	++++	++++	++++

* Expressed as the reciprocal of the dilution employed.
Range: ++++ = maximal growth.
− = no growth.

of extractions carried out between pH 3 and 9, it was observed that little variation in total activity was evident in the final preparation. A pH of 3.0 was arbitrarily selected for routine extractions, since this pH minimized problems with colloidal solutions. In a similar study on the influence of the extraction period on total yield of the tuberculostatic factor, it was found that the yield was approximately the same after either two or twenty-four hours of extraction at 4° C.

The following procedure was adopted for the extraction and isolation of the tuberculostatic principle.

Fifteen hundred grams of fresh bovine spleen were ground in a meat chopper and suspended in 3 liters of distilled water. Concentrated hydrochloric acid was added drop-wise with stirring until the pH of the mixture remained constant at pH 3.0. The extraction was continued overnight at 4° C. with constant stirring. The mixture was readjusted to pH 7.0 with saturated sodium hydroxide. The supernate was removed by centrifugation (3,000 g. for thirty minutes) and dialyzed for forty-eight hours against three changes of distilled water. The dialyzed supernate was adjusted to pH 5.0 with 1 N HCl which caused a flocculent precipitate to form. The precipitate was collected by centrifugation and dissolved in distilled water at pH 7.0. The dissolved protein was reprecipitated at pH 5.0 and redissolved at pH 7.0. This solution was extracted with ethyl ether for one hour with intermittent shaking, using a separatory funnel. The aqueous phase was recovered and subjected to an additional precipitation at pH 5.0. The precipitate was collected by centrifugation and dissolved in distilled water at pH 7.0 and lyophilized.

The material recovered after lyophilization was a light tan to white fluffy powder which was active against some strains of mycobacteria in concentrations ranging from 39 to 312 γ per ml. (table 2). The human strains of tubercle bacilli (Erdman and H37Rv) were found to be more susceptible to the inhibitor than the bovine strains (4228-4 and Ravenel). The attenuated BCG strain, however, showed the same susceptibility as the human virulent strains. *M. smegmatis* and *M. phlei* were completely resistant to the tuberculostatic factor in concentrations as high as 10 mg. per ml. Similar results were obtained in duplicate runs employing a medium without Tween 80.

Preliminary biochemical tests on this material suggested that it was protein in nature since it was nondialyzable and gave a positive biuret test and a negative

TABLE 2

THE TUBERCULOSTATIC ACTIVITY OF THE PURIFIED PROTEIN FROM BOVINE SPLEEN

Organism	Concentration of Protein in γ per Ml.							
	0	19	39	78	156	312	624	1248
M. tuberculosis BCG	++++	++++	++	–	–	–	–	–
M. tuberculosis H37Rv	++++	++++	++	–	–	–	–	–
M. tuberculosis Erdman	++++	++++	+++	–	–	–	–	–
M. tuberculosis Ravenel	++++	++++	++++	++++	++++	–	–	–
M. tuberculosis 4228-4	++++	++++	++++	++++	++	–	–	–
M. smegmatis	++++	++++	++++	++++	++++	++++	++++	++++
M. phlei	++++	++++	++++	++++	++++	++++	++++	++++

Range: ++++ = maximal growth.
– = no growth.

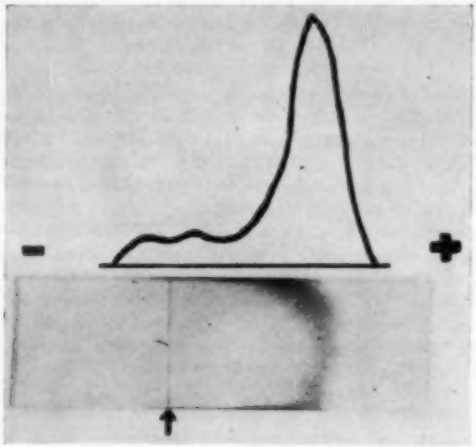

FIG. 1. Electrophoretic pattern of tuberculostatic protein employing paper electrophoresis.

alpha-naphthol test. A series of electrophoretic runs, employing a precision ionograph, were made to determine the purity and state of homogeneity of the tuberculostatic protein. A typical electrophoretic pattern reveals (figure 1) one relatively homogeneous peak as well as some trailing, which was usually encountered with paper electrophoresis. The protein component migrated to the anode at pH 8.6 indicating that it is negatively charged at this pH.

In a subsequent experiment the purified protein was applied to a series of ten

strips and ionographed for three hours (10 volts per cm.), at which time the strips were removed and each was cut into four uniform segments which were correspondingly pooled and eluted for thirty minutes in distilled water. The eluates obtained from the four preparations were sterilized by filtration through a Swinny filter and tested for tuberculostatic activity against the BCG strain. The eluate obtained from the segments corresponding to the location of the splenic protein was found to contain essentially all of the tuberculostatic activity. This was taken as evidence that the stainable protein component contained the active principle either in whole or in part.

Electrophoretic analysis was also carried out in veronal buffer pH 8.6, ionic strength 0.1 at a potential gradient of 7.5 volts per cm. in a Klett electrophoresis instrument. A typical result obtained with free electrophoresis is presented in figure 2, including the ascending and descending patterns. This figure illustrates a single boundary with a slight suggestion of some heterogeneity of this peak in the descending pattern.

Quantitative tests for carbohydrate, phosphorus, nitrogen, and desoxyribonucleic acid revealed that the material was largely protein in nature (table 3).

The ultraviolet absorption spectrum of the purified protein was determined with a Beckman model DU spectrophotometer (figure 3). The peak at 275 mu is

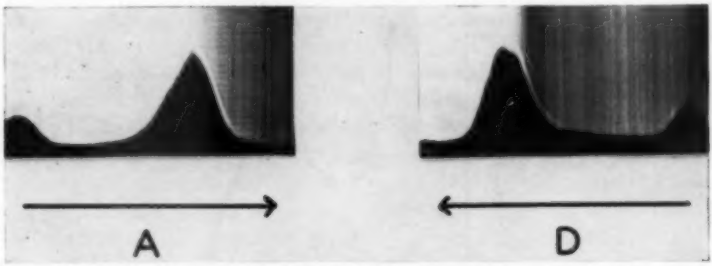

FIG. 2. Electrophoretic diagram of tuberculostatic protein under conditions of free electrophoresis. *A:* ascending pattern; *D:* descending pattern.

TABLE 3

CHEMICAL ANALYSIS OF TUBERCULOSTATIC PROTEIN FROM BOVINE SPLEEN

1. Total protein:	
a. Micro-Kjeldahl	89 per cent
2. Total nucleic acids:	
a. From total P	0.86 per cent
b. DNA by Dische Method	0.8 per cent
c. RNA by difference	0.06 per cent
3. Total carbohydrate:	
a. Anthrone method as glucose equivalents	0.9 per cent

P = phosphorus.
DNA = desoxyribonucleic acid.
RNA = ribonucleic acid.

consistent with the pattern found with substances containing aromatic amino acids.

Chromatography of the hydrolysate of the purified tuberculostatic protein revealed that glutamic acid, cystine, leucine, and alanine were present. A summary of the Rf values (table 4) provides additional data for the protein composition of the tuberculostatic substance in bovine spleen.

The pH at which minimal solubility occurred was determined by adjusting 5 fractions of a 0.1 per cent tuberculostatic protein solution with 1 N acetic acid to obtain the following pH values; 4.6, 4.7, 4.8, 4.9, 5.0, and 5.1. The tubes were allowed to stand at 4° C. for thirty minutes and were then read in a Coleman model 9 nephelometer. The tube adjusted to pH 4.8 produced the highest nephelometric reading, suggesting that the isoelectric point of this protein is approximately at this pH value. An exact determination of the isoelectric point by electrophoretic methods was not performed.

The heat stability of the active principle was studied by heating a 1 per cent aqueous solution of the protein at 56° C., 65° C., and 75° C. for periods up to two hours at pH 3.0, 7.0, and 8.5. It was observed that in all instances the tuberculostatic activity was destroyed within thirty minutes at 65° C. at pH 3.0. However, no loss of tuberculostatic activity was observed if the protein solution was heated up to two hours at 75° C. provided the pH was at 7.0 or 8.5.

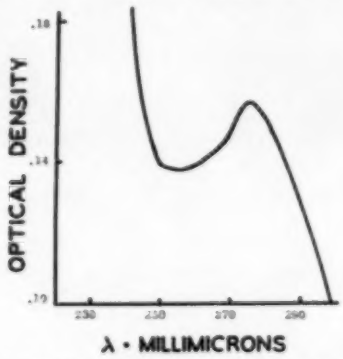

FIG. 3. Ultraviolet absorption pattern of tuberculostatic protein.

TABLE 4

CHROMATOGRAPHY OF HYDROLYZED TUBERCULOSTATIC PROTEIN ISOLATED FROM BOVINE SPLEEN

Amino Acids	Rf		Rf (Protein Hydrolysate)	
	Butanol-Acetic	Phenol	Butanol-Acetic	Phenol
Alanine	0.31	0.56	0.30	0.56
Cystine	0.06	0.20	0.08	0.19
Glutamic acid	0.20	0.28	0.22	0.27
Leucine	0.66	0.81	0.65	0.83

Additional experiments indicated that only a slight loss of activity was observed when the protein material was heated in a boiling water bath for periods up to two hours at pH 7.0.

In a series of experiments aimed at determining the optimal pH for activity of the tuberculostatic protein, it was observed that 156 γ per ml. were required to inhibit the H37Rv strain at pH 6.5, whereas between 39 and 78 γ per ml. were required to inhibit this strain at pH 7.3.

Discussion

The tuberculostatic protein isolated from bovine spleen is different from the classical tuberculostatic basic proteins isolated heretofore from various mammalian tissues. It migrates toward the anode at pH 8.6, indicating that the active principle is negatively charged at this pH value. The analytic determinations revealed that the purified material is low in carbohydrate and desoxyribonucleic acid, which suggests that protein constitutes the main class of material in the final preparation. This point finds additional support from the ultraviolet spectrum as well as from the results of chromatography of the protein hydrolysate.

Observations on the antibacterial activity of the splenic protein suggest that it is less active against virulent bovine tubercle bacilli than against virulent human tubercle bacilli and the attenuated bovine strain BCG. The significance of these data with regard to the resistance of cattle to human tubercle bacilli and their susceptibility to bovine tubercle bacilli is not clear. Since bovine tubercle bacilli appear to be more resistant than human tubercle bacilli to the inhibitory activity of such diverse compounds as ascorbic acid, reductones, and stearic acid, the differences in susceptibility to the tuberculostatic protein isolated from bovine spleen may not represent a significant factor in their virulence for the cow. Nevertheless, the pattern of tuberculostatic activity exhibited by the splenic protein may be a useful marker for investigating its role in resistance to tuberculosis. The complete resistance of *M. smegmatis* and *M. phlei* to the tuberculostatic protein revealed a striking specificity between the susceptibility of tubercle bacilli and saprophytes.

Tissue components which are inhibitory for tubercle bacilli could conceivably play a decisive role in suppressing multiplication of tubercle bacilli, especially in local areas of tissue necrosis. The protein isolated from bovine spleen and described in this paper may be unique in this regard since short extraction periods at a wide pH range succeeded in liberating the tuberculostatic principle. This tuberculostatic protein may represent a new class of tuberculostatic inhibitors.

Summary

A protein which displays tuberculostatic properties against virulent mammalian tubercle bacilli has been isolated from bovine spleen. It has an isoelectric point in the region of pH 4.8, is not dialyzable, is resistant to heating at pH 7.0, but is readily inactivated when heated at pH 3.0. It was somewhat more active against virulent human tubercle bacilli than against virulent bovine tubercle bacilli. It was inactive for two saprophytic strains (*M. smegmatis* and *M. phlei*).

Sumario

Nueva Proteína Tuberculostática Aislada del Bazo Bovino

Del bazo bovino se ha aislado una proteína que despliega propiedades tuberculostáticas contra los bacilos tuberculosos virulentos de mamífero. Muestra la misma un punto isoeléctrico en la zona de un pH de 4.8, no es dializable y es resistente a la calefacción a un pH de 7.0, pero se inactiva fácilmente al ser calentada a un pH de 3.0. Fué algo más activa contra los bacilos tuberculosos humanos virulentos que contra los bacilos tuberculosos bovinos virulentos. Resultó inactiva contra 2 cepas saprofitas (*M. smegmatis* y *M. phlei*).

Resume

Nouvelle protéine tuberculostatique isolée d'une rate bovine

Une protéine douée de propriétés tuberculostatiques contre le bacille tuberculeux de mammifère a été isolée à partir de rate bovine. Son point isoélectrique se situe aux environs de pH 4, 8; elle n'est pas dialysable; elle résiste à la chaleur à pH 7, mais à pH 3 elle est facilement inactivée par chauffage. Elle est légèrement plus active contre les bacilles humains virulents. Elle s'est révélée inactive contre les souches saprophytes (*M. smegmatis* et *M. phlei*).

Acknowledgment

The writers are grateful to Mr. Edward Berry of the Department of Biochemistry for studying the purified protein under conditions of free electrophoresis.

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Case Reports

MASSIVE DISSEMINATED INTRACRANIAL CALCIFICATION FOLLOWING "SEROUS TUBERCULOUS MENINGITIS"[1]

JOHN LORBER

(Received for publication February 13, 1958)

Introduction

As shown in another paper in this issue, intracranial calcification very frequently develops in children two years or more after tuberculous meningitis (1). In a series of 130 survivors, such calcifications were roentgenographically demonstrated in 63 cases (48.4 per cent). In a large majority of the patients, these calcifications developed in the basal meningeal exudate. In 17 cases (13 per cent), however, calcifications were demonstrated within the brain substance itself. Usually these intracerebral calcifications were small and single. Presumably they represent the small intracranial tuberculomata ("Rich foci") which are responsible for the development of tuberculous meningitis. It is known that such tuberculomata may give rise to a less widespread and nonfatal form of meningeal involvement (2), which Lincoln in 1947 called "serous tuberculous meningitis" (3).

Clinically this condition may show itself by a mild meningeal irritation which may occur in the course of an active tuberculous infection and may be indistinguishable from the earliest picture in true tuberculous meningitis. It may also be apparently silent and will be detected on routine examination of the cerebrospinal fluid in patients with acute miliary tuberculosis. The characteristics of the cerebrospinal fluid are an elevation of the cell count and occasionally of the protein, but the value for sugar remains normal and tubercle bacilli can only be demonstrated in exceptional instances.

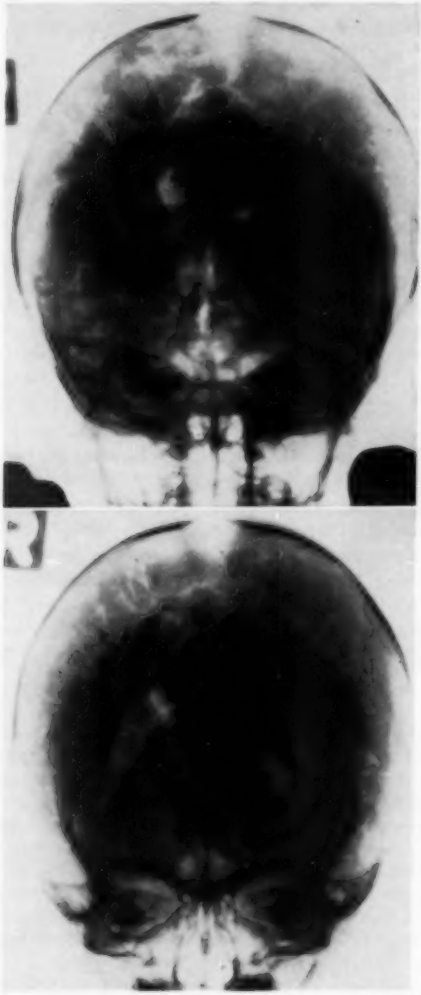
In view of the frequency of intracranial calcification following disseminated tuberculous meningitis, it was decided to carry out serial roentgenographic examination of the skulls of 14 children who recovered from "serous tuberculous meningitis" and survived for more than a year. No basal calcification was found in any, and intracerebral calcification was seen in only one patient. The calcification in this child, however, was so extensive and remarkable, and the case was so thoroughly investigated, that it warrants a separate report.

Case Report

R. G. was discovered to have primary pulmonary tuberculosis at the age of twenty-four months in May, 1952, when he was examined as a contact of his mother. He was symptom free at the time and was not given any specific treatment, but he was kept in the hospital under observation. He remained afebrile and symptom free until November 25, 1952. He then became ill, and a roentgenogram of his chest showed the typical "snowstorm" appearance of generalized miliary tuberculosis. On his admission to the Children's Hospital, Sheffield, in November, 1952, he was acutely ill. He had an early choroidal tubercle. Tubercle bacilli were seen in his gastric washings and were subsequently cultured and recovered from inoculated guinea pigs. These bacilli were fully susceptible to isoniazid, streptomycin, and PAS.

[1] From the University of Sheffield Children's Hospital, Western Bank, Sheffield 10, England.

The patient had no neck stiffness or other signs of involvement of the central nervous system but, in view of his miliary tuberculosis his cerebrospinal fluid was examined. At the first examination the cell count was 6 per cu. mm., the protein 50 mg. per 100 ml., and the sugar 61 mg. per 100 ml. In the course of the next thirteen months the cerebrospinal fluid was examined forty-three times. The sugar concentration remained invariably normal, and no tubercle bacilli were recovered by any method at any time. The cell count showed moderate fluctuations rising to a maximum of 28 per cu. mm. on two occasions within ten days



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FIGS. 1 and 2. Pneumoencephalograms showing displacement of the left ventricle downward and slightly toward and across the midline.

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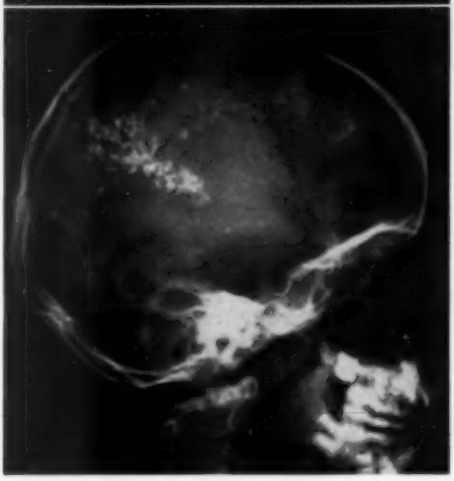

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FIGS. 3 and 4. Roentgenograms of the skull four years after admission, showing extensive scattered bilateral intracranial calcifications, with a large conglomerate mass in the left posterior parietal region.

of admission. It fell to normal on several occasions subsequently, but became persistently normal only after ten months. The protein remained persistently elevated throughout thirteen months, rising to a maximum of 280 mg. per 100 ml. on one occasion, two months after admission. At all other times it fluctuated between 50 and 140 mg. per 100 ml. These changes were considered to be consistent with the diagnosis of serous tuberculous meningitis.

The patient was being treated with streptomycin intramuscularly and with isoniazid and PAS orally, treatment being discontinued at the end of October, 1953. During his treatment for miliary tuberculosis, the patient's pulmonary condition improved and the miliary tubercles disappeared, but a major convulsion occurred at the end of December, 1952, and a series of convulsions occurred in March, 1953. This time a temporary left-sided papilledema of slight degree was present. It was presumed that the patient had a cerebral tuberculoma acting as a space-occupying lesion which was responsible for the persistent changes in the cerebrospinal fluid. A pneumoencephalogram performed in March, 1953, showed no evidence of hydrocephalus, but the left lateral ventricle was depressed and slightly displaced toward and across the midline (figures 1 and 2). An electroencephalogram was also performed. The tracing was within normal limits.

After the onset of his convulsions the boy was treated with phenobarbital and after March, 1953, no more convulsions occurred. The left-sided papilledema subsided completely. Up to the present, in January, 1958, the boy remains well and free from convulsions. He continues on prophylactic phenobarbital treatment.

Roentgenograms of the skull: In March, 1953 widening of the coronal sutures was found and was ascribed to raised intracranial pressure.

In January, 1954, fourteen months after the onset of the patient's "serous meningitis," faint but extensive calcified shadows became demonstrable. Serial roentgenograms subsequently showed that the shadows were progressively increasing in density and more and more appeared as time went by; but after 1956 no further change occurred. At this stage there were very large numbers of calcified shadows over the surface of the convexities of the brain on both sides as well as several scattered individual calcifications within the brain substance. One particularly large group was seen in the left posterior parietal region. (figures 3 and 4). The presence of this mass corresponded well with the presumed position of a space-occupying lesion demonstrated in the earlier pneumoencephalogram (*See* figures 1 and 2).

Discussion

A search of the literature (4) disclosed that the largest number of calcified intracranial tuberculomata so far described in one patient has been published by Weens (5). He confirmed his roentgenographic discovery by finding 11 calcified tuberculomata at necropsy.

The calcifications which developed in the present case were more extensive and more numerous than any paralleled in the literature, but another child was shown at a clinical meeting by Dr. Frances Braid in Birmingham whose history and the roentgenogram of whose skull were remarkably similar to those of the child just described.

It is interesting that such extensive intracranial lesions could be present without any neurologic symptoms or signs for so many years. It is probable, however, that the boy would have developed disseminated meningitis but for the antimicrobial treatment which he received during the active phase of his illness.

Summary

The case is described of a two-year-old boy who had generalized miliary tuberculosis associated with serous tuberculous meningitis. During the active phase of the disease and while receiving antimicrobial treatment, he had two episodes of major convulsions. A pneumoencephalogram at this stage revealed a displacement of the ventricular system.

Subsequently the boy made an uneventful recovery and remained free of convulsions and neurologic signs for five years. Nevertheless, roentgenograms of his skull revealed most extensive bilateral intracerebral calcification, especially in the left posterior parietal region.

Sumario

Calcificación Intracraneal Diseminada Masiva Consecutivamente a "Meningitis Tuberculosa Serosa"

Descríbese el caso de un niño de dos años que tenía granulia asociada con meningitis tuberculosa serosa. Durante la fase activa de la enfermedad y mientras recibía tratamiento antimicrobiano, el pequeño experimentó dos episodios de convulsiones mayores. Un neumoencefalograma reveló en esa ocasión desplazamiento del aparato ventricular. Después el niño se repuso sin complicsciones, permaneciendo exento de convulsiones y de signos neurológicos durante cinco años. A pesar de esto, las radiografías del cráneo revelaban calcificación intracerebral bilateral de lo más extenso, sobre todo en la región parietal posterior izquierda.

Resume

Calcification disséminée massive intracranienne consécutive à une "méningite tuberculeuse séreuse"

Description d'un cas concernant un petit garçon de 2 ans qui avait été atteint d'une tuberculose miliaire généralisée avec association de méningite tuberculeuse séreuse. Au cours de la phase active de la maladie, alors qu'il recevait un traitement antibactérien, il avait présenté deux crises de convulsions importantes. Le pneumoencéphalogramme effectué durant ce stade a révélé un déplacement du système ventriculaire. Ultérieurement l'enfant a guéri normalement et n'a présenté ni convulsions, ni signes neurologiques pendant cinq ans. Les radiographies du crâne ont néanmoins révéle une calcification intracérébrale bilatérale très étendue, en particulier dans la région pariétale postérieure du côté gauche.

Acknowledgment

The writer wishes to thank Dr. T. Lodge for the roentgenograms and Professor R. S. Illingworth for his criticism.

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BRONCHIAL PERFORATION AS A COMPLICATION OF BRONCHOSCOPY[1]

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Perforation of the trachea or the bronchus is a rare complication of bronchoscopy, and only a few reports on the subject have been published (1–7). Uncontrollable coughing and/or struggling by the patient or forceful instrumental manipulation are the usual causes. The endoscopist may discover the mucosal damage or the actual tear before withdrawal of the bronchoscope. Visualization of a perforation by fluoroscopy after intrabronchial injection of iodized oil has been reported by Samson (7). The clinical manifestations are those produced by air leakage from the respiratory tract, i.e., mediastinal and subcutaneous emphysema or pneumothorax. Pneumoperitoneum has also been encountered (5). Violent cough induced by intubation may sometimes cause a similar picture (1, 8). These findings, however, are much more likely to be due to instrumental airway perforation, and their occurrence following a bronchoscopy should be regarded as evidence of such a complication.

Post-bronchoscopic mediastinal and subcutaneous emphysema developed in 3 patients in a series of 2,184 bronchoscopies performed at Glen Lake Sanatorium over a thirty-year period (1926–1956). The first patient had tuberculous fibrostenosis of the right main bronchus into which a suction tube was passed "after some effort." The complication in the second patient apparently developed secondary to paroxysmal coughing during the procedure. In the third patient, in whom cough was a factor, a perforation was demonstrated on bronchograms made immediately after the endoscopy. This case is reported below.

CASE REPORT

A 43-year-old, white female was admitted to Glen Lake Sanatorium on March 10, 1955, with the diagnosis of bilateral, active pulmonary tuberculosis, moderately advanced. Chemotherapy with streptomycin and para-aminosalicylic acid was instituted in addition to the usual sanatorium regimen. The residual lesions in the apical and posterior segments of the right upper lobe were resected on November 16, 1955. Sputum cultures remained positive for tubercle bacilli, and resistance to streptomycin was demonstrated *in vitro*. The latter drug was, therefore, discontinued and replaced by isoniazid. On April 11, 1956, the patient underwent the combined procedures of bronchospirometry, bronchoscopy, and bronchography in preparation for excisional surgery on the left side. Following premedication with pentobarbital, atropine, and Pantopon®, topical anesthesia with 5 per cent cocaine was employed. The bronchospirometry and the first part of the bronchoscopy were without incident. However, when the orifice of the superior bronchus of the left lower lobe was being inspected, the patient began to cough, and the examination was completed with some difficulty. Bronchography with aqueous Dionosil® was then performed. She subsequently complained of cough, sore throat, and chest pain. The following morning, subcutaneous emphysema in the region of the neck, face, and anterior chest wall was present. Bronchograms made on the previous day were reviewed. A collection of opaque medium was noted in the mediastinum near the take-off of the superior branch of the left lower lobe bronchus (figure 1), apparently the result of leakage through a perforation in the bronchial wall. The patient was given penicillin and a cough depressant. The antituberculous drugs were continued. She remained afebrile except for a transient elevation of

[1] From Glen Lake Sanatorium, Oak Terrace, Minnesota.

temperature to 100.4° F. on April 13. A chest film taken on the same day revealed subcutaneous and mediastinal emphysema and the persistence of Dionosil in the mediastinum, although the bronchial tree was almost entirely cleared of the opaque material (figures 2, 3). The emphysema subsided in about a week. A roentgenogram taken on April 30 disclosed that the Dionosil, as well as the mediastinal and subcutaneous air, had been completely absorbed (figure 4). Thoracotomy and wedge resection of the apicoposterior segment of the left upper lobe were performed two weeks later. The bronchial tear had apparently healed. Recovery from the second operation was uneventful.

Comment

This is believed to be the first recorded case of bronchial perforation by the endoscope with bronchographic proof. The site of the tear and the pathway of the extravasated air are shown clearly on the roetngenograms.

Fig. 1. Bronchogram obtained on April 11, 1956. The white arrow indicates the collection of Dionosil in the mediastinum. The black arrow indicates the point of leakage near the take-off of the superior branch of the left lower lobe bronchus.

FIG. 2. Postero-anterior roentgenogram obtained on April 13, 1956. Note the persistence of Dionosil in the mediastinum (white arrow) and extravasated air (black arrows).

FIG. 3. Lateral roentgenogram obtained on April 13, 1956. Persistence of Dionosil in the mediastinum (white arrow).

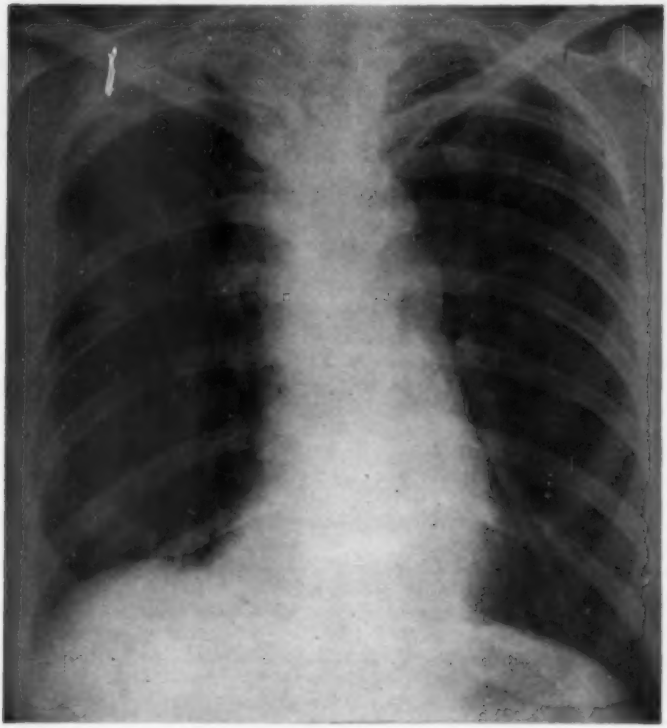

FIG. 4. Roentgenogram obtained on April 30, 1956. Note the complete absorption of Dionosil and extravasated air.

The importance of adequate sedation, meticulous topical anesthesia, proper positioning of the patient, and gentle instrumentation in the prevention of bronchial perforation cannot be overemphasized.

The clinical course may be mild, alarming (5), or fatal (1, 6). The severity of symptoms depends upon the location and size of the tear, the intraluminal and extraluminal pressure gradient, and the cardiopulmonary reserve of the patient. Suppression of cough and prophylactic antimicrobial therapy usually are sufficient in a mild case. Patients with severe symptoms may require oxygen, skin incisions for decompression, tracheotomy, mediastinotomy, or aspiration and drainage of air from pleural and peritoneal cavities (5).

It is noteworthy that manifest mediastinitis or empyema, either tuberculous or otherwise, was not observed in any of the reported cases. This includes the subject described, whose sputum was positive for tubercle bacilli at the time of the accident.

SUMMARY

A case of bronchial perforation secondary to bronchoscopy is reported. The perforation was demonstrated on bronchograms taken immediately after the procedure.

Sumario

Perforación Bronquial como Compilación de la Broncoscopia

Preséntase un caso de perforación bronquial secundaria a la broncoscopia. La perforación fué descubierta en los broncogramas tomados inmediatamente después del procedimiento.

Resume

Perforation bronchique comme complication de la bronchoscopie

Un cas de perforation bronchique succedant à une bronchoscopie. La perforation a été décelée sur les bronchogrammes qui ont été faits aussitôt après l'exploration.

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Notes

A RAPID METHOD FOR DETERMINING THE DRUG SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS

The isolation of *Mycobacterium tuberculosis* from clinical material and its growth on specific medium may require a period of two to six weeks. In many instances, an equal amount of time may be consumed in the determination of drug susceptibility after an isolated culture has been obtained. A rapid drug-susceptibility test for tubercle bacilli would be advantageous from many important aspects.

In a previous publication, Pital, Disque, and Leise described a rapid plate method for determining drug susceptibility.[1] In this plate test, resazurin was utilized as an oxidation-reduction indicator. For a number of years, resazurin has been employed for estimating the bacterial concentration of milk.[2-4] However, this indicator has not been used extensively in metabolic studies.[5-7] According to Twigg[5] and DeBaun and de Stevens[7] the reduction of resazurin may occur in two steps as follows:

$$\underset{\text{(blue)}}{\text{Resazurin}} \xrightarrow[-O]{} \underset{\text{(pink)}}{\text{Resorufin}} \underset{-2H}{\overset{+2H}{\rightleftharpoons}} \underset{\text{(colorless)}}{\text{Dihydroresorufin}}$$

The present report describes a rapid (five to eight days) susceptibility test for *M. tuberculosis*, employing resazurin as the oxidation-reduction indicator.

The test organisms used in this study were *M. tuberculosis* strains H37Ra and H37Rv. Bacterial suspensions were prepared for testing by removing 2 loopfuls (3 mm. diameter loop) of fresh growth from a Petragnani slant, and inoculating 75 ml. of Dubos broth (Difco) containing 0.02 per cent Tween® 80 and 0.5 per cent albumin. In order to facilitate dispersion of bacterial clumps, the broth medium was dispensed into a sterile, 1,000-ml. Erlenmeyer flask containing glass beads which were 4 mm. in diameter. A sufficient number of beads were added to cover the entire bottom of the flask. After introduction of the test organism, the flask was tightly sealed and placed on a reciprocating shaker (100 strokes per minute, 3-inch stroke) for two hours. Such treatment resulted in the formation of a finely dispersed bacterial suspension. After shaking, the bacterial density of each suspension was measured in the Coleman Universal spectrophotometer at 550 mu. Undiluted samples from each flask were used for optical density measurements. As a safeguard against possible contamination, penicillin and actidione were added to the homogenized suspension. Final concentrations of penicillin and actidione were 40 units per ml. and 125 γ per ml., respectively. These suspensions were then allowed to incubate for twenty-four hours at 37°C. After incubation, 5 ml. were dispensed into 50-ml. Erlenmeyer flasks containing previously prepared discs of dihydrostreptomycin or isoniazid. These discs were of appropriate concentration to yield 0.006, 0.06, 6.0, and 60.0 γ per ml. when contained in a 5 ml. volume. In several experiments, 12.0 γ discs were also prepared. Each drug concentration was tested in du-

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plicate. The drug-containing discs were prepared by immersing sterile, blank paper discs[8] (6.35 mm. in diameter and with an absorbing capacity of 0.03 ml.) in aqueous solutions of each drug. The discs were dried and stored at refrigeration temperature.

The test organism was incubated in the presence of dihydrostreptomycin for four days; a period of seven days was required with isoniazid. Following the prescribed period of incubation, 0.7 ml. of 0.05 per cent resazurin solution was added to each flask. The dye solution was prepared by bringing 100 ml. of phosphate buffer pH 7.0 to a boil, and adding 5 resazurin tablets[9] (10.8 mg. per tablet). The solution was thoroughly shaken and cooled prior to being used. The flasks with dye were placed on a fluorescent light-box containing a ground glass window and were incubated at 37°C. for an average period of five hours. The use of a light-box was essential for observing color changes during incubation. The drug-susceptibility results were recorded when control flasks without drug were reduced to a definite pink. Inhibitory drug concentrations prevented the reduction of resazurin from blue to pink by tubercle bacilli. Resazurin was reduced to purple and finally to pink in control flasks as well as in flasks containing noninhibitory concentrations of drug. A more persistent purple stage of dye reduction was evident when a particular drug concentration exerted a slight inhibitory effect. However, the purple stage was eventually reduced to pink.

In order to ensure that contaminants were not present, samples from representative flasks were streaked on heart-infusion agar plates, and stained preparations were examined microscopically for the presence of acid-fast bacilli. Upon completion of each test, flasks were incubated for four days in order to determine the duration of inhibition by the drug.

A comparative study was made between the rapid resazurin method and a tube dilution test. In both procedures, identical concentrations of drug were employed as well as the same test strain (H37Ra). Tube dilutions of each drug were prepared in Dubos broth containing Tween 80 and albumin. Quadruplicate tubes were used for each concentration, and the final volume was adjusted to 5 ml. Each tube was inoculated with 0.05 ml. of a 1:10 dilution of a thirteen-day broth culture. The tubes were sealed and incubated for sixteen days at 37°C. The drug susceptibility was estimated by the presence or absence of growth.

Control experiments were conducted to determine whether or not viable cells were necessary for dye reduction by adding resazurin to bacteria-free filtrates of suspensions and broth cultures as well as to sterile Dubos broth, and incubating for seven days at 37°C.

The rapid resazurin method was also evaluated in the presence and absence of Tween 80. *Mycobacterium tuberculosis* strain H37Rv was used as the test organism. The test medium consisted of Dubos broth without Tween 80 (Difco) and 0.5 per cent albumin.

Effective concentrations of dihydrostreptomycin impaired the resazurin-reducing capability of tubercle bacilli within four days. On the other hand, a total of seven days was required with isoniazid.

Identical drug-susceptibility results were obtained with *M. tuberculosis* H37Ra and and H37Rv. The same concentrations of dihydrostreptomycin and isoniazid (6 and 60 γ per ml.) were inhibitory for both strains (table 1). However, there was a distinct difference in the duration of inhibition for both drugs at concentrations of 6 and 12 γ per ml. (table 2). Twenty-four hours after completion of each test, inhibition with isoniazid was slight at 6 and 12 γ per ml., and completely absent within forty-eight hours. On the other hand, complete inhibition with dihydrostreptomycin was still evident after ninety-six hours. The inhibitory effect of 60 γ of isoniazid per ml. was comparable to that of dihydrostreptomycin.

The presence or absence of Tween 80 did not appear to affect the drug-susceptibility

[8] Obtained form Schleicher and Schuell, Keene, New Hampshire.

[9] Obtained from the National Aniline Division of the Allied Chemical and Dye Corp., New York, New York.

TABLE 1

THE DRUG SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS AS DETERMINED BY THE RESAZURIN REDUCTION METHOD

Test Strain	Number of Tests	Optical Density Range	Resazurin Reduction				
			Drug γ/ml*				
			0.006	0.06	6.0	60.0	Control
			Dihydrostreptomycin				
H37Ra	5	0.125–0.174	+	+	−	−	+
H37Rv	3	0.097–0.143	+	+	−	−	+
			Isoniazid				
H37Ra	5	0.107–0.181	+	+	−	−	+
H37Rv	3	0.108–0.155	+	+	−	−	+

* Each concentration tested in duplicate.
+ Reduction of resazurin to pink (no inhibition).
− Failure to reduce resazurin (inhibition).

TABLE 2

DURATION OF INHIBITION OF MYCOBACTERIUM TUBERCULOSIS (H37Ra and H37Rv) FOLLOWING COMPLETION OF DRUG SUSCEPTIBILITY TESTS

Drug	Concentration γ/ml*	Hours of Incubation			
		24	48	72	96
		Resazurin Reduction			
Isoniazid	6	±	+	+	+
	12	±	+	+	+
	60	−	−	−	−
	Control	+	+	+	+
Dihydrostreptomycin	6	−	−	−	−
	12	−	−	−	−
	60	−	−	−	−
	Control	+	+	+	+

* Duplicate concentrations examined in all test series.
+ Reduction of resazurin to pink (no inhibition).
− Failure to reduce resazurin (inhibition).
± Reduction of resazurin to purple (slight inhibition).

results obtained by the rapid resazurin technique. Identical results were also obtained with the rapid resazurin technique and a tube dilution test (table 3). The results with the tube dilution test were available in a total of twenty-nine days following growth of the test culture, whereas only five and eight days, respectively, were required for dihydrostreptomycin and isoniazid, utilizing the experimental methods described in the present report.

The prolonged incubation of cell-free filtrates and sterile Dubos broth in the presence

TABLE 3

THE SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS (H37Ra) TO DIHYDROSTREPTOMYCIN AND ISONIAZID AS DETERMINED BY THE RAPID RESAZURIN METHOD AND A TUBE DILUTION TEST

Test Method	Drug	Total Time of Test	Resazurin Reduction or Growth*				
			Drug γ/ml†				
			0.006	0.06	6.0	60.0	Control
		Days					
Rapid resazurin test	Dihydrostreptomycin	5	+	+	−	−	+
	Isoniazid	8	+	+	−	−	+
Tube dilution test	Dihydrostreptomycin	29	+	+	−	−	+
	Isoniazid	29	+	+	−	−	+

* Resazurin method: Inhibition indicated by failure of test organism to reduce resazurin. Tube dilution test: Inhibition indicated by failure of test organism to grow.

† All concentrations tested in quadruplicate.

of resazurin revealed that these substances did not possess any detectable reducing activity. In this test, the intact, viable cell was apparently necessary for dye reduction.

Addition of 2 loopfuls of culture material to 75 ml. of broth produced an optical density range of 0.097 to 0.181 as determined in the Coleman Universal spectrophotometer. A marked increase in cell numbers (3 to 4 loopfuls), represented by optical densities of more than 0.200, proved to be too overwhelming for testing susceptibility to isoniazid. A more concentrated cell suspension (optical density of more than 0.200), however, could be employed with dihydrostreptomycin.

* * *

The primary objective of the present study was the development of a rapid as well as a routine test method for determining the drug susceptibility of *M. tuberculosis*. The adaptation of this procedure to a routine test was considered from three important aspects: (*1*) preparation of a test bacterial suspension from freshly grown cultures on solid medium; (*2*) attaining the desired concentrations of drug without resort to tedious dilution techniques; and (*3*) estimation of drug susceptibility on the basis of metabolic inhibition rather than growth.

In the preparation of a test suspension, the addition of 2 loopfuls of culture material to 75 ml. of broth produced a range of bacterial densities that was optimal for rapid susceptibility testing (table 1). Therefore, from the standpoint of a routine test procedure, the measurement of optical density or adjustment of suspensions to a fixed density could be eliminated. It must be emphasized that freshly grown cultures were employed for the drug-susceptibility studies. Cultures should be tested as soon as sufficient growth (2 loopfuls) appears on slants or plates. Consistent and rapid results were always obtained with young cells. On the other hand, reducing activity became erratic and markedly diminished in those cultures incubated beyond eight weeks or stored in the refrigerator for longer than two to three months. It should also be mentioned that the volume of test materials can

be reduced so that a lesser amount of inoculum is required. For example, several drug concentrations could be eliminated and, therefore, only one loopful of growth would be required for the preparation of 37.5 ml. of broth suspension.

The use of drug-impregnated discs provided a convenient method for obtaining desired drug concentrations without the necessity of preparing various dilutions each time a drug-susceptibility test was conducted. In the present study, two inhibitory and two non-inhibitory drug concentrations were used. A stock supply of discs could be prepared in any desired range of concentrations.

In a tube dilution test, inhibition of tubercle bacilli is usually determined by the presence or absence of visible growth or, more accurately, by turbidity measurements. The initial inoculum is comparatively small in the tube dilution test, and a prolonged period of incubation may be required before final end-point readings can be recorded. In the rapid resazurin procedure, the broth medium is seeded in bulk with a heavier inoculum, and drug-susceptibility is determined on the basis of metabolic inhibition rather than on growth. The use of a rapid oxidation-reduction indicator obviated the necessity for turbidity measurements, since inhibition could be demonstrated by a simple color change. The elimination of turbidity readings was considered essential for routine work involving a large number of tests.

Interference with the microbial reduction of resazurin by the drug revealed different patterns for both dihydrostreptomycin and isoniazid. For example, inhibition could be demonstrated much earlier with dihydrostreptomycin than with isoniazid. Furthermore, isoniazid concentrations of 6.0 and 12.0 γ per ml. inhibited resazurin reduction for a comparatively short period of time, whereas a persistent inhibition occurred at the 60.0 γ concentration. In contrast, the same concentrations of dihydrostreptomycin completely inhibited resazurin-reducing activity for the duration of the experimental study. Transient inhibition with inadequate drug concentrations could also be demonstrated. These patterns of inhibition probably reflect different modes of drug action. Similar results with isoniazid and streptomycin have been obtained by other investigators employing broth dilution preparations or various plating techniques.[10-12] However, results with these procedures are usually available only after a prolonged period of time, whereas similar information may be furnished by the rapid resazurin method within five to eight days. It was of interest to note that identical drug-susceptibility results were obtained with the rapid resazurin method and a tube dilution test. This observation suggests that inhibition of metabolism and growth are directly related, although metabolic inhibition can be detected much earlier with proper experimental techniques.

Finally, the test procedure described in this report may be used for the rapid *in vitro* screening of potential antituberculous drugs. A large number of compounds could be evaluated within a relatively short period of time.

In summary: A rapid drug-susceptibility test for *Mycobacterium tuberculosis* was developed. Interference with microbial reduction of resazurin was used as the criterion for estimating the susceptibility to the drug. After growth of the test culture on a solid medium the susceptibility to dihydrostreptomycin was determined in five days, while a period of eight days was required for isoniazid. Test strains H37Ra and H37Rv were found to be susceptible to 6.0 and 60.0 γ per ml. of dihydrostreptomycin and isoniazid. A comparative study of the rapid resazurin method and a tube dilution test yielded identical results with

[10] Mackaness, G. B., and Smith, N.: Am Rev. Tuberc., 1953, *67*, 322.
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[12] Barclay, W. R., *et al.*: Am. Rev. Tuberc., 1953, *67*, 490.
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Mycobacterium tuberculosis H37Ra. However, results with the tube test were available only four weeks after growth of the test culture. Drug interference with the reducing capability of tubercle bacilli rapidly revealed different patterns of inhibition for dihydrostreptomycin and isoniazid. The use of the resazurin test method for rapid screening of potential antituberculous drugs is suggested.

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October 24, 1957

STUDIES ON SERUM LIPASE

The diagnosis of tuberculosis without discovering tubercle bacilli, either in the sputum or in any other tuberculous material, is dependent solely on clinical and roentgenographic evidence which at times is not pathognomonic of the disease. A biochemical or serologic test, if available, would be of very great value in establishing the diagnosis in doubtful cases.

The chemical test involving determination of serum lipase has its origin in the finding that the tubercle bacillus has a lipoid membrane—a fact that has been confirmed by electron microscopic study.[1] Moreover, Metalinikov has offered the explanation that virulent strains of *M. tuberculosis* fail to kill or induce the disease in a caterpillar, *Galleria mellonella*, because of the high lipase content in the caterpillar.[2] Porter[3] has reported a high serum lipase level in cats, which are found to be very resistant to tuberculosis; whereas the guinea pig, which has a very low serum lipase, is most susceptible to tuberculosis. Rogers[4] found a decrease in serum lipase in tuberculosis as well as in leprosy compared with the serum lipase of normal subjects. From all of these observations it might be postulated that a host not rich in lipase may become susceptible to tuberculosis because of inability to dissolve the lipoid covering of the invading tubercle bacilli. It is conceivable that such an alteration of the surface of the bacillary cell might result in impairment of the phagocytic mechanism.

Middlebrook[5] has reported that tubercle bacilli secrete a substance which inhibits or destroys the lowered lipase. This in turn would result in lowering of the serum lipase level in active tuberculosis. Seabra[6] has claimed that the lipase index of serum is of greater diagnostic value in early pulmonary tuberculosis than roentgenography. The present study was undertaken to see if such a claim is justified.

The lipase index was estimated in patients with pulmonary and extrapulmonary tuberculosis in different stages of the disease, in patients suffering from nontuberculous chest disease, and in normal persons.

Five to 7 ml. of fasting blood were withdrawn by venepuncture and allowed to clot. Clear serum was separated and used for the determination of the lipase index. Fatty acids split off from a cottonseed oil emulsion by incubating with serum were titrated against standard sodium hydroxide and the titer value was used as an index of the lipase content.

Reagents

(*1*) *Oil emulsion:* Five grams of acacia (USP) were dissolved in distilled water and the pH was adjusted to 5.4 with 1 per cent sodium carbonate. To this were added 100 ml. of cottonseed oil (pure and having an acidity lower than 0.25 per cent), drop by drop in a Waring Blendor. The emulsion was then placed in vacuum to draw off the dissolved gases, filled in glass ampules, and sterilized.

(*2*) *Standard sodium hydroxide:* N/30 checked frequently.

(*3*) *Buffer:* Potassium phosphate, monobasic, AR—8.31 gm.; sodium phosphate, 12 H_2O dibasic AR—7.37 gm.; distilled water to make up to 100 ml.

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[1] Rosenblatt, M. B., *et al.*: Am. Rev. Tuberc., 1942, *46*, 587.

[2] Metalinikov, S.: L'Infection Microbienne et l'Immunité chez la Mite des Abcilles *Galleria Mellonella*, Masson et Cie., Paris, France.

[3] Porter, A. E.: Biochem. J., 1916, *10*, 523.

[4] Rogers, L.: Lancet, 1924, *1*, 1297.

[5] Middlebrook, G.: Am. Rev. Tuberc., 1945, *51*, 244.

[6] Seabra P.: Zeitschr. Hyg., 1954, *139*, 121.

The final pH was adjusted to 6.5. To 5 ml. of the above given buffer, 23 ml. of 1:1,000 merthiolate solution were added and the volume was made up to 600 ml. with distilled water.

(4) *Alcohol indicator solution:* Neutral absolute ethyl alcohol containing 1.2 ml. of 1 per cent phenolphthalein.

Procedure

Buffered substrate was prepared by adding 4 ml. of buffer to every ml. of oil emulsion in a 25-ml. conical flask, with thorough shaking.

To a 25-ml. conical flask were added 5 ml. of buffered substrate and 1 ml. of clear serum, stoppered, and thoroughly shaken. To another flask, 1 ml. of distilled water was added instead of serum. This served as a blank.

The flasks were incubated at 37°C. for twenty-four hours. At the end of this period, 5 ml. of alcohol indicator were added and titrated against N/30 sodium hydroxide. The difference in the ml. of sodium hydroxide required for the test and the blank multiplied by ten gave the lipase index.

In table 1 are given the number of patients examined, their mean lipase index, together with standard deviation and standard error of the mean, and the frequency distribution in all patients examined.

The percentage of patients with lipase index less than a particular value for different groups is given in table 2. For comparison the lipase index in 18 normal healthy subjects is also shown in the table. In table 3 it is indicated whether the differences observed in the lipase index between any two groups in all of the above-mentioned cases are statistically highly significant (*SS*), significant (*S*), or not significant (*NS*).

From the results obtained it may be observed that the lipase index differs significantly between the early and advanced stages of pulmonary tuberculosis. The lipase index appears to decrease with the progress of the disease; hence it will be of prognostic value.

TABLE 1

FREQUENCY DISTRIBUTION OF SERUM LIPASE INDICES FOR PATIENTS WITH TUBERCULOSIS, OTHER CHEST DISEASES, AND FOR NORMAL SUBJECTS

Serum Lipase Index	Active Pulmonary Tuberculosis		Pulmonary Tuberculosis, Arrested	Pulmonary Tuberculosis: All Active Cases	Non-pulmonary Tuberculosis	Tuberculosis: All Cases	Chest Diseases, Nontuberculous	Normal Healthy Subjects
	Duration							
	Less than 6 Months	More than 6 Months						
0–2	14	9	1	23	3	26	12	—
2–4	12	17	1	29	4	33	20	—
4–6	17	7	3	24	1	25	23	1
6–8	4	—	2	4	2	6	15	7
8–10	4	2	4	6	2	8	6	5
10–12	3	—	3	3	—	3	7	2
12–14	2	1	—	3	—	3	5	3
Total	56	36	14	92	12	104	88	18
Mean index	4.93	3.3	7.05	3.97	3.67	3.93	5.53	9.06
Standard deviation	3.12	1.43	3.57	3.04	2.6	2.99	3.7	2.6
Standard error of the mean	0.42	0.24	0.92	0.32	0.75	0.29	0.37	0.62

TABLE 2

PERCENTAGE OF PATIENTS WITH LIPASE INDICES OF LESS THAN A PARTICULAR VALUE

Serum Lipase Index	Active Pulmonary Tuberculosis		Pulmonary Tuberculosis, Arrested	Pulmonary Tuberculosis: All Stages	Nonpulmonary Tuberculosis	Tuberculosis: All Cases	Chest Diseases, Nontuberculous	Normal Healthy Subjects
	Duration							
	Less than 6 Months	More than 6 Months						
Less than 2.....	25	25	7	25	25	25	14	—
Less than 4.....	46	72	13	57	58	57	36	—
Less than 6.....	77	92	33	83	67	81	62	5.6
Less than 8.....	84	97	46	87	83	87	80	44
Less than 10.....	91	100	73	93	100	97	86	72
Less than 12.....	96	—	93	97	—	100	94	83
Less than 14.....	100	—	100	100	—	—	100	100

TABLE 3

RESULTS OF STATISTICAL ANALYSIS OF THE DIFFERENCES IN LIPASE INDICES

Normal	Active Tuberculosis, Less than 6 Months	Active Tuberculosis, More than 6 Months	Arrested Tuberculosis	Nonpulmonary Tuberculosis	Tuberculosis: All Forms	Nontuberculous Chest Diseases
Normal						
Active tuberculosis, less than six months	SS					
Active pulmonary tuberculosis, more than six months	SS	S				
Arrested tuberculosis	NS	SS	SS			
Nonpulmonary tuberculosis	SS	NS	NS	SS		
Tuberculosis, all forms	SS	—	—	SS	—	
Nontuberculous chest diseases	SS	S	SS	NS	S	SS

SS = statistically highly significant.
S = significant.
NS = not significant.

Wolf,[7] while studying the serum lipase level in tuberculous patients had noticed that the serum lipase level in patients having bad prognoses was diminished. A similar observation was made by Desbordes and German,[8] using a different method for determining the lipolytic enzyme activity. They noticed that the enzyme activity was diminished most in those who had advanced progressive tuberculosis of long duration.

* * *

The highly significant difference in the lipase index found in the present studies in patients with active tuberculosis, on one hand, and in patients whose disease was arrested, on the other, agrees well with the studies made by the above-mentioned writers. In the present studies there was no significant difference in the lipase index of pulmonary and nonpulmonary tuberculosis.

When the lipase index of patients with nontuberculous chest disease was compared with

[7] WOLF, J. E.: J.A.M.A., 1939, *112*, 677.

[8] DESBORDES, J., AND GERMAN, A.: Bull. Soc. chim. biol., 1944, *26*, 509.

that of those who had tuberculosis for less than six months, the difference was significant; when compared with that of patients with advanced tuberculosis for more than six months, the difference was highly significant. The difference was also highly significant when compared with tuberculosis as a whole and irrespective of the duration.

In order to find out whether the lowering of lipase index was specific to tuberculosis, lipase indices in patients suffering from tuberculous and nontuberculous chest disease were compared with those in normal persons of comparable age. A glance at table 2 shows that 94 per cent of the normal subjects had lipase indices of more than 6 units compared with 19 per cent of the tuberculous patients. The remaining 81 per cent had indices of less than 6 units. Moreover, 57 per cent of tuberculous patients had indices of less than 4 units compared with 37 per cent of the nontuberculous patients. This means that more than one-third of the nontuberculous patients could not be distinguished from the tuberculous patients. Hence, the value of the lipase index test in the diagnosis of early tuberculosis is not statistically significant. This finding is in accordance with the views of Hangleiter and Reuter[9] who noticed a lowering of lipase index in many pathologic conditions, such as carcinoma or hepatic or gall bladder disease. However, the present study, showing a decrease in lipase index along with progress of the disease, is not in conformity with the study by Krant and Burger[10] who did not observe any such difference.

In summary: Serum lipase index determinations were made in patients with tuberculosis, nontuberculous chest diseases, and in normal subjects. The technique adopted for the determination was the same as that suggested by Seabra. Statistical analysis showed a significant fall in the lipase index with the progress of the disease. Hence, the test may be of prognostic value. Statistical comparison showed that there is no significant evidence to indicate that the lipase determination is of value in the diagnosis of early tuberculosis, particularly when applied to any single individual.

It was not possible to confirm the observations of Seabra that the lipase test is a convincing guide in the diagnosis of tuberculosis. The lipase index is lowered in a certain number of patients suffering from other chest diseases, and there is wide variation in serum lipase values both in normal subjects and among patients with tuberculosis.

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[9] HANGLEITER, H., AND REUTER, A.: Ztschr. ges. exper. med., 1940, *107*, 355.
[10] KRANT, H., AND BURGER, H.: Ztschr. physiol. chem., 1938, *253*, 105.

THE MECHANISM OF THE COMBINED EFFECT OF ANTITUBERCULOUS DRUGS

Numerous reports have been made on the combined effect of antibacterial drugs. The mechanism of the *in vitro* combined effect, however, has been discussed by only a few investigators. It appears that the mechanism of the combined effect might be discussed from the following two points of view: The combined effect on a single cell and the combined effect on a bacterial population.

If a drug can significantly modify cellular metabolism, although not inhibit cell division, it is possible that two drugs acting on a single cell may together effect complete inhibition when each alone could not. This possibility has been discussed by Mudd.[1] It is also possible that two drugs acting on a single cell may antagonize each other. This possibility has been discussed by Szybalski and Bryson[2] and by Klein.[3]

With respect to the effect on a bacterial population, if the mutation rate to a drug resistance and the mutation rate to another drug resistance are of 1×10^{-m} and 1×10^{-n}, respectively, the mutation rate to double resistance would be of $1 \times 10^{-(m+n)}$, that is, extremely low. Survivors would presumably be reduced to extremely low levels by the simultaneous use of two drugs. This possiblity has been discussed by Klein and his coworkers.[4-6]

These two points of view with respect to the combined effect are associated with two observations of the action of a drug on the growth of bacteria. The action of a drug on a population as a whole would be to influence the number of viable cells which are members of the original population, and the action of the drug on a single cell would consist of influencing the growth rate of individual viable cells. The purpose of the present paper is to observe the mechanism of the combined effect from these two points of view.

Mycobacterium tuberculosis var. *hominis*, strain H37Rv, was employed in this study. Ogawa's egg medium was employed throughout the study, its composition is as follows: basal solution (1 per cent sodium glutamate and 1 per cent KH_2PO_4), 100 ml.; whole eggs, 200 ml.; glycerol, 6 ml.; 2 per cent malachite green, 6 ml. The medium was poured in 8 ml. quantities into a test tube, 170 by 17 to 18 mm. and was sterilized at 90° C. for sixty minutes. Dihydrostreptomycin sulfate,[7] isoniazid,[8] and sodium para-aminosalicylate[8] were employed. These drugs were dissolved in distilled water and added to the medium before sterilization. The activity of dihydrostreptomycin was considered to be one-half of the original concentration because of the decreased activity of the drug resulting from absorption of the drug to coagulated egg protein.

Method for determining the number of survivors on various media: Six-week-old cultures growing as discrete colonies were shaken with glass beads for ten minutes and suspended with saline. Tenfold dilutions were made of the suspension. Various dilutions thus made were inoculated in 0.02 ml.-quantities to each tube of three sets consisting of various concentrations of single drugs or various combinations of two drugs. Inoculation was made with a whirled loop which transferred 0.02 ml. The method for inoculating gives less varia-

[1] Mudd, S.: J. Bact., 1945, *49*, 527.
[2] Szybalski, W., and Bryson, V.: Am. Rev. Tubere., 1953, *68*, 280.
[3] Klein, P.: Deutsch. med. Wchnschr., 1954, *79*, 445.
[4] Klein, M., and Kalter, S. S.: J. Bact., 1945, *51*, 95.
[5] Klein, M., and Kimmelman, L. J.: J. Bact., 1947, *54*, 363.
[6] Klein, M., and Schorr, S. E.: J. Bact., 1953, *65*, 454.
[7] Manufactured by Meiji Co.
[8] Manufactured by Shionogi Co.

tion of bacterial numbers than the drop method or the pipetting method of inoculating.[9] Counts were made after a five-week incubation period at 37°C. Counts of a set giving less than 150 colonies per tube were utilized for the basis of calculation. The number of colonies was considered to be the number of survivors, although it must be realized that the number of colonies does not always give the number of survivors. For, even when all individual cells could be viable, any colonies might be not viable because of an extremely retarded growth rate.

Method for determining the growth rate: The colony diameter was measured after a six-week incubation period on a set of media inoculated by a tenfold more diluted suspension than the suspension employed for determining the number of survivors, and it was considered to be an index of the growth rate. The growth rate would be more accurately given by the generation time. The generation time has been determined on various media and the results obtained will be reported in another paper.

As mentioned above, the bacterial population utilized in the present study contained less than 150 viable cells. Accordingly, a probability that the population contained any mutants is negligible and, therefore, variation of the drug susceptibility among individual cells of the population would be considered to be the physiologic variation (fluctuation) of susceptibility occurring among cells bearing the susceptible genotype.

Drug concentrations utilized in the present study were 0.5 to 2 γ of streptomycin per ml. of medium, 0.005 to 0.01 γ of isoniazid per ml. of medium, and 0.05 to 0.1 γ of PAS per ml. of medium. As shown in the results, some of these concentrations did not significantly affect the number of survivors. The drug concentrations utilized would be considered, therefore, to be so-called "subinhibitory" or "partially inhibitory." Under the same conditions as given here, a complete inhibition of the visible growth after a six-week incubation period has been given by 5 γ of streptomycin, 0.03 γ of isoniazid, and 0.8 γ of PAS, respectively.

The combined effect on the number of survivors: The results obtained are shown in tables 1 and 2. If there were no specific interaction between two drugs, it would be expected that the ratio of the number of survivors on medium containing two drugs (c) is a product of the ratios on medium containing single drugs (a and b); that is, a correlation $c = ab$ would exist. For example, the ratio of the number of survivors on medium containing 0.5 γ of streptomycin is 95.5 per cent and its 95 per cent confidence limits are 78.6 to 116 per cent; the ratio on medium containing 0.1 γ of PAS is 65.7 per cent and its 95 per cent confidence limits are 49.2 to 85.5 per cent. From these values, the ratio of the number of survivors on medium containing both 0.5 γ of streptomycin and 0.1 γ of PAS is expected to be $0.955 \times 0.657 \times 100$ per cent $= 62.8$ per cent; and its 95 per cent confidence limits are expected to be $0.786 \times 0.492 \times 100$ to $1.16 \times 0.855 \times 100$ per cent $= 38.5$ to 99.0 per cent. On the other hand, the ratio estimated is 45.3 per cent and its 95 per cent confidence limits are 31.1 to 62.0 per cent, as shown in table 1. Therefore, there is no significant difference between the ratio expected and the ratio estimated.

As shown in table 2, no significant difference has been observed between the ratios expected and those estimated on various combinations of two drugs. Therefore, under the conditions tested, the correlation $c = ab$ would seem to be true, and no significant interaction seems to exist between these three drugs, streptomycin, isoniazid and PAS. However, the result does not exclude the presence of interaction between two of the drugs. Indeed, it appears possible that a specific correlation may exist between the action of streptomycin and that of PAS, for a tendency for the ratios estimated to be smaller than those expected has been observed in most combinations of streptomycin and PAS, as shown in table 2, although each of the differences was not statistically significant.

[9] Tsukamura, M., and Noda, Y.: Kekkaku, 1957, *32*, 639 (in Japanese).

TABLE 1

RATIOS OF SURVIVORS ON VARIOUS MEDIA

Medium	Number of Colonies per Tube		Ratios of Survivors: Mean and Its 95% Confidence Limits‡
	Mean ± SD*	95% Confidence Limits†	
γ/ml.			*per cent*
No drug (control)	114.8 ± 12.7	105.7–123.9	100.0
SM 0.5	110.0 ± 17.6	97.5–122.5	95.5 (78.6–116)
SM 1	60.5 ± 26.7	41.5–79.5	52.8 (33.5–75.5)
SM 2	61.5 ± 27.4	42.0–81.0	53.6 (34.0–76.7)
INH 0.005	100.0 ± 24.2	82.8–117.2	87.1 (66.8–111)
INH 0.01	63.9 ± 13.9	54.0–73.8	55.6 (43.6–70.0)
P 0.05	109.1 ± 16.9	97.1–121.1	96.0 (78.3–114)
P 0.1	75.5 ± 20.3	61.0–90.0	65.7 (49.2–85.5)
SM 0.5-P 0.05	127.6 ± 16.6	111.0–144.2	111 (89.5–136)
SM 0.5-P 0.1	52.0 ± 18.8	38.6–65.4	45.3 (31.1–62.0)
SM 1-P 0.05	64.8 ± 27.5	45.2–84.4	56.5 (36.4–80.0)
SM 1-P 0.1	30.5 ± 10.2	22.2–37.8	26.5 (17.9–35.9)
SM 2-P 0.05	22.3 ± 8.18	16.4–28.2	19.4 (13.2–26.8)
SM 2-P 0.1	10.2 ± 4.20§	7.20–13.2	8.80 (5.72–12.5)
SM 0.5-INH 0.005	117.2 ± 11.0	109.3–125.1	102 (88.0–119)
SM 0.5-INH 0.01	118.8 ± 19.0	105.3–132.3	104 (85.0–125)
SM 1-INH 0.005	61.9 ± 12.3	53.1–70.7	54.0 (42.8–67.1)
SM 1-INH 0.01	49.1 ± 9.12	42.6–55.6	42.7 (34.4–52.5)
SM 2-INH 0.005	82.0 ± 15.6	70.9–93.1	71.5 (57.2–88.2)
SM 2-INH 0.01	70.8 ± 21.1	55.8–85.8	61.6 (45.0–81.5)
INH 0.005-P 0.05	106.2 ± 35.2	81.1–131.3	92.5 (65.5–124)
INH 0.005-P 0.1	125.0 ± 33.5	101.2–148.8	108 (81.5–141)
INH 0.01-P 0.05	70.5 ± 23.2	53.9–87.1	61.5 (43.5–82.5)
INH 0.01-P 0.1	50.2 ± 17.2	38.0–62.4	43.7 (30.6–59.3)

SM = streptomycin; INH = isoniazid; P = PAS

* Mean of 10 replicates and standard deviation

† Derived from "t" × (Standard Error)

‡ Derived from the following equation: (Mean) = B/A; (95% confidence limits) = $B1/A2---B2/A1$. Here, $A(A1---A2)$ is the mean number of colonies and its 95 per cent confidence limits (minimum---maximum) on media containing no drug and $B(B1---B2)$ is that on media containing drugs.

§ Not accurate because of too small size of colonies

No significant antagonism has been observed under the above conditions, although Szybalski and Bryson[2] have reported an antagonism between isoniazid and streptomycin under conditions different from those utilized in the present studies.

Combined effect on the growth rate: As has been mentioned previously, the colony diameter would be considered to be an index of the growth rate. The results obtained are shown in tables 3 and 4. The colony diameter may not always be an accurate index of the growth rate. As shown in table 3, a reduction in the colony diameter has been observed on medium containing isoniazid. Colonies on this medium, however, were more raised than those on medium containing no drug. The total amount of growth of the former appeared not to be reduced compared with that of the latter; that is, the growth rate on medium containing isoniazid did not appear to be retarded. Indeed, the generation time on medium contain-

TABLE 2

COMPARISON OF THE RATIOS OF SURVIVORS EXPECTED AND ESTIMATED IN COMBINATIONS OF TWO DRUGS GIVING A SIGNIFICANT DIFFERENCE FROM THE RATIOS ON MEDIA CONTAINING SINGLE DRUGS

Combination	Expected	Estimated
γ/ml.	*per cent*	*per cent*
SM 0.5-P 0.1	62.8 (38.5-99.0)	45.5 (31.1-62.0)
SM 1-P 0.05	50.1 (26.3-86.0)	56.5 (36.4-80.0)
SM 1-P 0.1	34.6 (16.4-64.5)	26.5 (17.9-35.9)
SM 2-P 0.05	50.8 (26.6-87.5)	19.4 (13.2-26.8)
SM 2-P 0.1	35.1 (16.7-65.6)	8.89 (5.72-12.5)*
INH 0.01-P 0.1	36.5 (21.4-60.0)	43.7 (30.6-59.3)

SM = streptomycin; INH = isoniazid; P = PAS

* Not accurate because of too small size of colonies

TABLE 3

RATIOS OF COLONY SIZES ON VARIOUS MEDIA

Medium	Diameter of Colonies		Ratios of Colony Diameters: Mean and Its 95% Confidence Limits†‡
	Mean ± SD*	95% Confidence Limits†	
γ/ml.	*mm.*		*per cent*
No drug (control)	2.11 ± 0.337	1.95-2.27	100.0
SM 0.5	2.09 ± 0.511	1.85-2.33	99.0 (81.5-120)
SM 1	2.06 ± 0.424	1.86-2.26	97.5 (82.0-116)
SM 2	1.10 ± 0.505	0.96-1.34	52.0 (42.2-68.8)
INH 0.005	1.74 ± 0.576	1.47-2.01	82.5 (64.8-103)
INH 0.01	1.86 ± 0.520	1.62-2.10	88.0 (71.5-108)
P 0.05	0.750 ± 0.181	0.665-0.835	35.5 (29.3-42.9)
P 0.1	0.468 ± 0.148	0.399-0.537	22.2 (17.6-27.5)
SM 0.5-P 0.05	0.468 ± 0.271	0.341-0.595	22.2 (15.0-30.5)
SM 0.5-P 0.1	0.288 ± 0.082	0.249-0.327	13.6 (11.0-16.8)
SM 1-P 0.05	0.407 ± 0.219	0.304-0.510	19.3 (13.4-26.2)
SM 1-P 0.1	0.269 ± 0.100	0.222-0.316	12.7 (9.80-16.2)
SM 2-P 0.05	0.250 ± 0.053	0.225-0.275	11.8 (9.90-14.1)
SM 2-P 0.1	ca. 0.1 or less		
SM 0.5-INH 0.005	2.03 ± 0.301	1.89-2.17	96.5 (83.5-111)
SM 0.5-INH 0.01	2.06 ± 0.448	1.85-2.27	98.0 (81.5-116)
SM 1-INH 0.005	1.90 ± 0.246	1.78-2.02	90.0 (78.5-104)
SM 1-INH 0.01	1.88 ± 0.436	1.67-2.09	89.0 (73.5-107)
SM 2-INH 0.005	1.17 ± 0.409	0.978-1.37	55.5 (43.2-70.5)
SM 2-INH 0.01	1.29 ± 0.518	1.04-1.54	61.0 (45.8-79.0)
INH 0.005-P 0.05	0.780 ± 0.331	0.626-0.934	34.5 (27.5-48.0)
INH 0.005-P 0.1	0.670 ± 0.174	0.588-0.752	31.7 (26.0-38.7)
INH 0.01-P 0.05	0.579 ± 0.207	0.482-0.676	27.5 (21.2-34.6)
INH 0.01-P 0.1	0.287 ± 0.106	0.237-0.337	13.6 (10.4-17.3)

SM = Streptomycin; INH = isoniazid; P = PAS

* Mean of 20 replicates and standard deviation.

† Derived from "t" × (Standard Error).

‡ Derived from the following equation: (Mean) = B/A; (5% confidence limits) = $B1/A2 - - - B2/A1$. Here, $A(A1 - - - A2)$ is the mean diameter of colonies and its 95 per cent confidence limits (minimum - - - maximum) on media containing no drug and $B(B1 - - - B2)$ is that on media containing drugs.

TABLE 4

COMPARISON OF THE RATIOS OF COLONY DIAMETERS EXPECTED AND ESTIMATED IN COMBINATIONS OF TWO DRUGS GIVING A SIGNIFICANT DIFFERENCE FROM THE RATIOS ON MEDIA CONTAINING SINGLE DRUGS

Combination	Expected	Estimated
γ/ml.	*per cent*	*per cent*
SM 0.5-P 0.05	35.2 (23.9–51.5)	22.2 (15.0–30.5)
SM 0.5-P 0.1	22.0 (14.3–33.0)	13.6 (11.0–16.8)
SM 1-P 0.05	34.5 (24.0–49.9)	19.3 (13.4–26.2)
SM 1-P 0.1	21.6 (14.4–31.9)	12.7 (9.80–16.2)
SM 2-P 0.05	18.5 (12.4–29.5)	11.8 (9.90–14.1)
SM 2-P 0.1	11.4 (7.43–19.0)	ca. 5
INH 0.01-P 0.1	19.6 (12.6–29.7)	13.6 (10.4–17.3)

SM = Streptomycin; INH = isoniazid; P = PAS

ing the same concentrations of isoniazid has been indicated to be almost the same as on medium containing no drug. On the other hand, the generation time on medium containing streptomycin and that on medium containing PAS have been indicated to be much more retarded than that on medium containing no drug. The results will be reported in another paper. The results shown in tables 3 and 4 would indicate that the same correlation $c = ab$ exists with respect to the colony diameter as with respect to the number of survivors, when the ratio of the colony diameter on medium containing two drugs is designated as c and the ratios on medium containing single drugs as a and b.

A tendency for the colony diameter estimated to be smaller than that expected has been observed in all of the combinations of streptomycin and PAS tested, although the difference in each case has been statistically insignificant. It appears very probable, therefore, that a specific interaction exists between the actions of streptomycin and PAS on the growth rate, and the growth rate on medium containing both streptomycin and PAS is more significantly retarded than would be expected from the retarding effect of single drugs. Such tendency has not been observed in combinations of streptomycin and isoniazid or of isoniazid and PAS except in one case.

The effect of a drug, A or B, in reducing the number of survivors or the colony diameter would be expressed as $(1 - a)$ or $(1 - b)$ and the effect of both drugs, C, as $(1 - c)$. This is based on the supposition that the ratios of the number of survivors or the colony diameter as a and b on medium containing single drugs and c on medium containing both drugs. (Here, the ratio on medium containing no drug is taken as the unit.) Accordingly, the effect of a drug is $A = 1 - a$ and the effect of another drug is $B = 1 - b$, and the effect of the two drugs is $C = 1 - c$. Substituting these for the equation $c = ab$, the following correlation is obtained: $C = A + B - AB$. Therefore, under the conditions tested, the combined effect of two drugs would be expressed as the difference between the product and the addition of the effects of single drugs.

* * *

The combined effect of streptomycin, isoniazid and PAS on *Mycobacterium tuberculosis* has been analyzed from two points of view in order to observe the action of drugs: (*1*) the action affecting the number of survivors of a bacterial population, which are starting members of the bacterial population; and (*2*) the action affecting the growth rate of a single cell.

In the present report, the combined effect of the drugs in so-called "subinhibitory" or

"partially inhibitory" concentrations not affecting most individual viable cells, or affecting a part of the individual viable cells, has been tested on a bacterial population of *Mycobacterium tuberculosis* var. *hominis* that has fluctuating variations in drug susceptibility.

Under the conditions tested, it has been indicated that the ratio of the number of survivors or the colony diameter, which is an index of growth rate, on medium containing two drugs is practically expressed as a product of the ratios of the number of survivors or the colony diameter on medium containing each single drug, when the ratio on medium containing no drug is taken as the unit. That is, the following equation exists: $c = ab$. Here, c is the ratio of the number of survivors or the colony diameter on medium containing two drugs, and a and b are the ratios on medium containing each single drug, when the ratio on medium containing no drug is taken as the unit.

From the results, it has been indicated that the combined effect of two drugs, C, may be practically expressed as the difference between the addition and the product of two effects of single drugs under the conditions tested. That is, when the effects of two single drugs are designated as A and B, respectively, the following equation exists: $C = A + B - AB$.

A tendency for the number of survivors and the colony diameter (that is an index of the growth rate) to be smaller on medium containing both streptomycin and PAS than those calculated from the above equation ($c = ab$) has been observed in most of the experiments, however, this difference between the estimated and the calculated colony size has remained statistically insignificant in each case.

It has been suggested that almost complete inhibition of the visible growth on medium containing "subinhibitory" or "partially inhibitory" concentrations of streptomycin and PAS may result from doubling the retarding effects of these two drugs on the growth rate of *Mycobacterium tuberculosis*.

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November 13, 1957

[10] The writers wish to express their appreciation to Dr. Rokuro Katsunuma, Director of the Obuso National Sanatorium; and to Professor Susumu Hibino, Nagoya University, for their help in the conduct of this study.

EFFECT OF TRANQUILIZING DRUGS ON HOSPITALIZED TUBERCULOUS PATIENTS

The adjustment of a patient to hospitalization and the acceptance of long-term treatment for tuberculosis poses many problems for which tranquilizing agents should be very beneficial. Many patients need help only for a short period of time, but others have trouble constantly throughout their hospitalization.

The evaluation of tranquilizing drugs clinically is a very difficult problem since many patients change from week to week in their general feelings and concern about their own personal problems. Thus, in many cases, the value of sedative or tranquilizing drugs is impossible to evaluate. Because of the need of a drug to help patients in a tuberculosis hospital, however, some attempt at evaluation of some of the tranquilizing agents was thought to be desirable. A small pilot study was therefore undertaken with the realization that effects would have to be fairly obvious or evaluation of the drug would be impossible.

Fifteen male patients were chosen for this study. All of the patients had been in the hospital at least three months prior to the study. These patients were chosen intentionally for their lack of cooperation on the division. They were all restless, apprehensive, uncooperative in their exercise requirements, and required both daytime and nighttime sedation in some form. Two weeks prior to initiation of the study, all sedative and tranquilizing drugs were discontinued. As a group the patients were then placed on the study for a period of two weeks with each of five different drugs. The drugs were indistinguishable one from the other. All were prescribed as one capsule three times a day and at bedtime. Thus, the total study period lasted for twelve weeks, with ten weeks on drug therapy and a week before and after treatment for observation.

During the two observation periods and during the period of treatment, all patients were observed daily by one of the writers (V. E.), the head nurse, the occupational therapist, and a social worker. Daily notes were made on each patient by these observers according to ten major criteria as indicated in table 1.[1] Comparison of the patient's condition before, during, and after the study was analyzed to determine positive or negative response to each drug. In addition, comments were also noted regarding side effects which could possibly be related to drug therapy.

At the end of the study period all results were compiled from the various observers and recorded, as in table 2. Since minimal worsening of the patient's mental attitude was frequently indistinguishable from mild side reactions to the drugs, no attempt was made to separate these. The results were therefore compiled on the basis of moderate to severe side reactions, minimal worsening of the mental status or minimal side effects and as no change, minimal benefit, moderate benefit, and marked benefit in attitude or mental status.

The drugs were administered as a double blind study and the present report was written and completed before the names of the drugs were known to the writers. The drug names were then substituted in the rough-draft stage. Medication consisted of five different capsules as follows: (*a*) two placebos, (*b*) phenaglycodol (Ultran[2]), 300 mg.; (*c*) meprobamate,[2] 400 mg.; and (*d*) ethchlorvynol (Placidyl[3]), 200 mg. Evaluation results are recorded in table 1.

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[1] Shatin, L., and Freed, E. X.: J. Ment. Sc., 1955, *101*, 444.

[2] Ultran and meprobamate were supplied through the courtesy of Dr. J. M. Maas of Eli Lilly & Company.

[3] Placidyl was supplied through the courtesy of Dr. George H. Berryman of Abbott Laboratories, Inc.

TABLE 1

FORM OF OBSERVERS' NOTES ON PATIENT

Name	Sex	Age	Diagnosis	Medication	Dosage	Basic Mental Status Previous to Study	Date			
							4-22	4-23	4-25	4-26
K. R.	M	28	Pulmonary Tuberculosis	No. 4	1 capsule three times daily and at bedtime					
1. The patient sleeps well.						+	+	++	+++	+++
2. The patient has good or bad mood.						±	0	0	+	+
3. The patient asks for pass.						−	0	0	0	0
4. The patient has to be pushed to follow routine.						±	0	0	0	−
5. The patient is interested in occupational therapy.						+	0	0	++	0
6. The patient often volunteers information about himself.						−	0	+	−	±
7. The patient is either silent or talks foolishly during visits.						+	±	−	±	−
8. The patient is irritable and grouchy.						±	0	0	0	0
9. The patient is cooperative.						±	0	0	0	0
10. The patient accepts hospitalization.						±	0	0	0	+

Yes or good, +; fair, ±; no or poor, −; no change, 0.

TABLE 2

EVALUATION OF TRANQUILIZING DRUGS ON DOUBLE-BLIND TECHNIQUE

Medication		Placebo	Phenaglycodol	Placebo	Ethchlorvynol	Meprobamate
Side effect	Moderate or severe side reaction	1	2	9	7	0
	Worsening or minimal side reaction	3	5	18	9	2
Patient attitude and cooperation	No change	21	24	9	0	19
	Minimal improvement	4	4	0	12	3
	Moderate improvement	4	4	0	7	0
	Marked improvement	0	0	0	10	0

Drug No. 1, placebo[4-6] *(four times a day):* The observers believed that the majority of patients received no benefit from this drug. One patient experienced mild anorexia, drowsiness, and abdominal discomfort, and refused to take the drug for two days. When therapy was reinstituted, the same symptoms reappeared. This patient, however, was intolerant to many different drugs and it was impossible to separate the psychogenic

[4] REITAN, R. M.: Antibiot. Med. & Clin. Therap., 1957, *4*, 158.
[5] WOLF, S., AND PINSKY, R. H.: J.A.M.A., 1954, *155*, 339.
[6] KOTEEN, H.: Ann. Int. Med., 1957, *47*, 978.

influence in this case. Likewise, several patients from time to time stated that they were able to sleep better, but this was not a consistent finding and could again be the result of suggestibility on the part of the patient.

Drug No. 2, phenaglycodol[1, 4, 7, 16] *(300 mg. four times a day):* While on this drug, one patient developed a papular rash over the face and anterior thorax and noted malaise. After one week of treatment, drug therapy was discontinued and the rash and malaise disappeared. Several patients complained of drowsiness, dizziness, and lack of ability to concentrate early in the course of treatment with this drug, and in several patients this persisted throughout the two-week period. There was some abdominal discomfort on the part of several of the patients, with occasional nausea, loss of appetite, and mild gastrointestinal symptoms. The majority of the patients, however, showed no significant change in their cooperation and mental status.

Drug No. 3, placebo[4-6] *(four times a day):* During administration of this capsule many of the patients experienced abdominal cramps, diarrhea, sweating, and anorexia. Three patients refused medication because of the gastrointestinal symptoms, and, in addition, many complained from time to time of headache, dizziness, and drowsiness. Their cooperation and mental attitude remained unchanged, however, and none seemed to benefit from the drugs. On the contrary, the increase in various complaints added to the difficulty of controlling the patients.

Drug No. 4, ethchlorvynol[8-12, 17] *(200 mg. four times a day):* All patients complained of dizziness and drowsiness one-half to one hour following the administration of the drug for the first four to five days. Some patients complained of numbness of the face and blurring of vision. One patient refused the drug because of these symptoms but, in those who continued to take the drugs, the side effects seemed to diminish as treatment continued. Several of the patients requested more of the medication and without exception they stated "I feel drunk." During the latter part of the period of administration of this drug, symptoms were limited to slight dizziness. All of the patients experienced definite improvement in their mental status and cooperation while on this drug. The degree of improvement varied, but it seemed to be quite consistent.

Drug No. 5, meprobamate[4, 12-15] *(400 mg. four times a day):* There were no significant changes or side reactions in the patients during the administration of this drug.

* * *

After evaluation of these impressions and before the type of medication was known, it was the conclusion of the investigators that: capsule *No. 1* and capsule *No. 5* represented control medication (placebo); capsule *No. 2* and capsule *No. 3* had produced no beneficial effects on the patients' cooperation and mental attitude; and that capsule *No. 4*

[7] Bernstein, C., and Klotz, S. D.: J.A.M.A., 1957, *163*, 930.

[8] New and Nonofficial Remedies, 1957: Evaluated by the Council on Pharmacy and Chemistry.

[9] Current Concepts in Therapy: Sedative-hypnotic drugs: I, New England J. Med., 1956, *255*, 520.

[10] McLamore, W. M., *et al.*: J. Organic Chem., 1955, *20*, 109.

[11] P'an, S. Y., *et al.*: J. Pharm. & Exper. Therap., 1955, *114*, 326.

[12] Rein, C. R., and Fleischmajer, R.: A.M.A. Arch. Dermat., 1957, *75*, 438.

[13] Osinski, W. A.: Ann New York Acad. Sc., 1957, *67*, 766.

[14] Dixon, N. M.: Ann. New York Acad. Sc., 1957, *67*, 772.

[15] Selling, L. S.: J.A.M.A., 1955, *157*, 1594.

[16] Slater, I. H., *et al.*: Proc. Soc. Exper. Biol. & Med. (in press).

[17] Lasagna, L.: J. Chronic Dis., 1956, *3*, 122.

was the only drug which produced definite benefit, but at the same time was accompanied by rather uncomfortable side effects. Even with these side reactions, however, only one patient refused further medication with this drug and the rest of the patients requested more medication in spite of the side reactions. It is of interest that capsule *No. 3* (placebo) was accompanied by gastrointestinal symptoms which, of course, must have been psychogenic in origin or, more likely, unrelated to the medication.

In summary: The evaluation of tranquilizing drugs is an extremely difficult problem. Complicated questionnaires and evaluation procedures have been used, with various results. The present study tends to support the clinical impression that many of the tranquilizing drugs are of no real value in the doses ordinarily prescribed and certainly offer little to the tuberculous patient who is posing a management problem. Difficulty of evaluation poses many problems in the use of these tranquilizer drugs. The need for a good drug of this type in the tuberculous patient is certainly present. Obvious clear-cut benefit will have to be present before such drugs can be accepted for such use.

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SEROMUCOID (SERUM MUCOPROTEIN) VALUES IN PATIENTS UNDERGOING HINCONSTARCH THERAPY[1]

The values for seromucoid are known to be raised in patients with active tuberculosis,[2, 3] as in other infectious diseases. Turner and associates[2] suggested that routine seromucoid estimations would probably be a valuable aid in the management of pulmonary tuberculosis. Hitherto, however, no study of the variations in seromucoid values during the course of chemotherapy has been reported. An opportunity was presented during the clinical investigation of hinconstarch[4] to examine the seromucoids in 55 patients and to follow the values in detail in 19 patients.

Estimations were made at weekly intervals during the first four weeks after the start of treatment, then fortnightly or monthly as the condition of the patients improved. Analyses of the sera were carried out within twenty-four hours of venipuncture whenever possible. Otherwise, sera were stored at 0°C. Seromucoid was determined in duplicate, on serum deproteinized by perchloric acid, by the method of Edward,[5] which is based on the colorimetric determination of "sialic acid." An empiric curve was made with dried seromucoid, isolated from normal serum by the method of Winzler and co-workers,[6] and results were expressed as mg. of seromucoid per 100 ml. of serum. Values determined in 30 apparently healthy subjects ranged between 100 and 140 mg. per 100 ml. The figures quoted in the earlier paper[5] for the normal range were too high, due to an arithmetic error.

A comparison of the seromucoid values with the extent of infiltration and cavitation at the start of treatment in 20 patients is given in table 1. There is a rough correlation between the amount of disease and the serum analysis, and all patients with fever had very high values for seromucoid (more than 490 mg. per 100 ml.). Any roentgenographic improvement was reflected in the lowering of seromucoid values. Closure of cavities was usually preceded by a return to normal seromucoid values, although in 6 patients (Nos. *23*, *35*, *39*, *42*, *48*, and *49*)[7] the seromucoid concentration became normal without cavity closure sixteen, sixteen, twelve, twenty-one, thirty-three, and forty-nine weeks, respectively, after the start of therapy. (*Note:* All of these 6 patients showed disappearance of the tubercle bacilli from the sputum.)

The results of sputum examinations and seromucoid estimations in 55 patients are given in figure 1. There appears to be good correlation between the presence of tubercle bacilli in the sputum and the value for seromucoid. In 11 of 19 patients studied in detail, seromucoid values returned to normal within two weeks (plus or minus) of the time when the disappearance of tubercle bacilli from the sputum had been demonstrated (fortnightly examinations). In 2 other patients whose sputum remained infectious, the seromucoid values remained high. Two other patients had normal values for seromucoid four weeks before sputum "conversion," and another had normal seromucoid values five months before the bacilli had disappeared from the sputum. The last-named patient

[1] The writer carried out this investigation while holding a Lasdon Foundation Research Fellowship.

[2] Turner, G. C., *et al.*: Dis. of Chest., 1953, *23*, 154.

[3] Winzler, R. J.: In *Methods of Biochemical Analysis*, Interscience, New York, 1955, p. 279.

[4] Barry, V. C., *et al.*: Am. Rev. Tuberc., 1958, 77, 952.

[5] Edward, D. W.: Irish J. M. Sc., 1956, *363*, 130.

[6] Winzler, R. J., *et al.*: J. Clin. Invest., 1948, *27*, 609.

[7] For fuller details of these patients *see* Barry *et al.*, Am. Rev. Tuberc., 1958, 77, 952.

TABLE 1

COMPARISON OF ROENTGENOGRAPHIC EXTENT OF DISEASE AND THE SEROMUCOID VALUES IN 20 PATIENTS AT THE COMMENCEMENT OF THERAPY

Seromucoid Value (*mg./100 ml. of serum*)									
140–190		190–250		250–300		300–400		400 and More	
I	C	I	C	I	C	I	C	I	C
1	0	3	3	3	1	3	0*	4	1*
2	0	4	4	1	2	2	2	3	3*
2	2	4	4	2	2	2	3	3	3*
				2	2	3	3	5	4*
				3	3				
				4	4				

I = infiltration; 1 = minimal; 6 = involving all three zones of both lungs (other figures intermediate).

C = cavitation; 0 = none; 4 = multiple large (other figures intermediate).

* Patient pyrexial at time blood sample was taken.

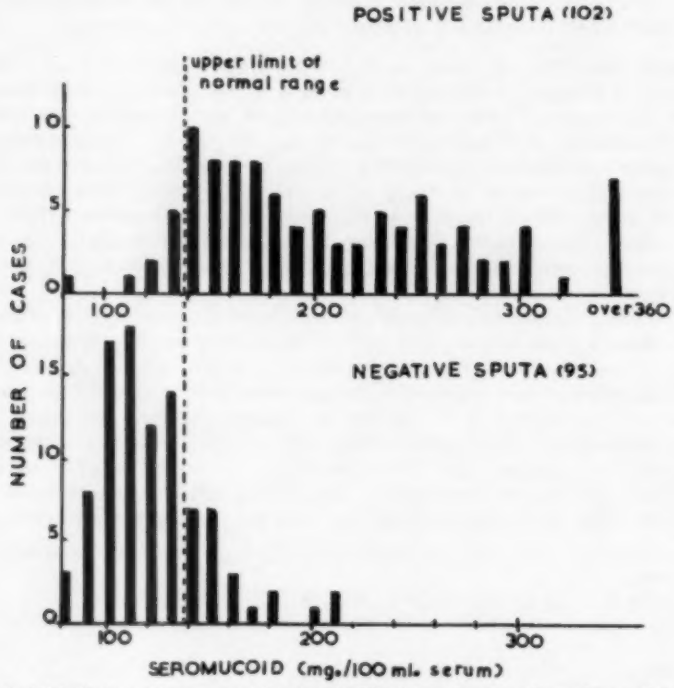

FIG. 1. Distribution of seromucoid values in 197 determinations in patients with roentgenographic evidence of tuberculosis.

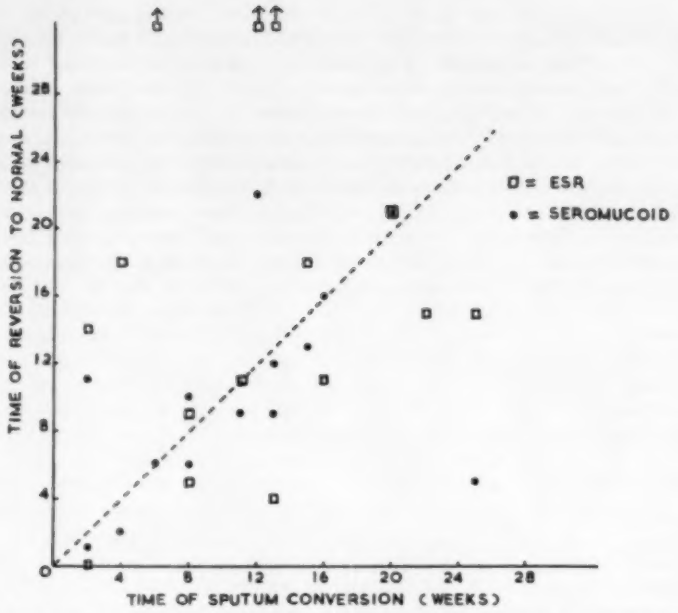

FIG. 2. Comparison of the times of the reversion of the erythrocyte sedimentation rate and seromucoid values to normal values with the time of disappearance of tubercle bacilli from the sputum.

(No. *60*) excreted organisms resistant to 1γ of isoniazid per ml. *ab initio*. In 3 patients, reversal of sputum infectiousness was attained six to eight weeks before the seromucoid values became normal.

The times when the erythrocyte sedimentation rate and seromucoid values reverted to normal are compared with the time of reversal of infectiousness in 15 patients (figure 2). It may be seen that seromucoid reversion time correlates better with the disappearance of tubercle bacilli from the sputum than does the erythrocyte sedimentation rate "reversion time." In two other patients, whose sputum remained infectious, and in one other whose sputum became noninfectious later, the erythrocyte sedimentation rate fluctuated considerably, reaching normal values at times, whereas the seromucoid values remained elevated. Throughout this series, the seromucoid values and the erythrocyte sedimentation rate varied independently. This corresponded with the findings of Seibert and associates,[8] who showed that total serum glycoprotein values were not directly related to changes in the sedimentation rate in tuberculosis.

The observation that normal seromucoid values are present while there is still roentgenographic evidence of considerable disease indicates that the seromucoids are not directly related to the extent of damaged tissue. However, the finding that the return of seromucoid values to normal paralleled reversal of sputum infectiousness suggests that seromucoid values may be considered as a measure of the activity of the disease proc-

[8] SEIBERT, F. B., *et al.*: J. Clin. Invest., 1947, *26*, 90.

ess. As bacteriologic cultures require at least two weeks, routine seromucoid analysis would frequently give prior indication of disappearance of tubercle bacilli from the sputum. In interpreting seromucoid values, however, it must be remembered that many other pathologic conditions with tissue breakdown or proliferation, such as surgical or accidental trauma[9, 10] rheumatoid arthritis or cancer,[11, 12] also increase the seromucoids, whereas parenchymatous liver disease may reduce the values below the normal range.[13, 14]

In summary: In a series of patients having moderately or far advanced pulmonary tuberculosis and treated with hinconstarch, it was found that the values for seromucoid showed a rough correlation with the degree of disease present and that roentgenographic improvement was reflected in a lowering of the seromucoid values. A good correlation was obtained between the disappearance of tubercle bacilli from the sputum and the return to normal values for the seromucoids.

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[9] WINZLER, R. J., AND BURKE D.: J. Nat. Cancer Inst., 1944, *4*, 417.
[10] EDWARD, D. W.: PH.D. Thesis, University of Birmingham, England, 1954.
[11] SHETLAR, M. R., *et al.*: J. Lab. & Clin. Med., 1956, *48*, 194.
[12] SHETLAR, M. R., *et al.*: J. Clin. Invest., 1953, *32*, 1208.
[13] GREENSPAN, E. M. *et al.*: J. Lab. & Clin. Invest., 1952, *39*, 44.
[14] MANDEL, E. E., *et al.*: Clin. Chem., 1955, *1*, 221.

RESULTS OF A CLINICAL TRIAL OF NEOMYCIN AEROSOL IN THE TREATMENT OF PULMONARY TUBERCULOSIS

Neomycin has been reported to be effective against *M. tuberculosis*[1] but has been found too toxic[2] to be used parenterally. A group of investigators[3] recently used neomycin as an aerosol and noted clinical improvement in 43 of 84 patients with pulmonary tuberculosis. In some cases the treatment produced roentgenographic evidence of improvement and disappearance of tubercle bacilli from the sputum. The greatest improvement was described in the recent cases, but a favorable response also occurred in 14 of 47 patients whose lesions were more than two years old. No toxicity other than the occurrence of oral moniliasis was reported. The absence of toxicity was believed to be due to the low concentration of neomycin found in the blood when the drug was used as an aerosol. The dosage used most often was equivalent to 0.5 gm. of neomycin sulfate in 2 to 4 ml. of water, to which was added 1 ampule of hyaluronidase. The number of daily doses varied between two and four, depending on the severity of the tuberculosis in each patient.

The present report describes an attempt to reproduce the above findings. Fourteen patients at this hospital have been given a trial of neomycin[4] aerosol treatment. The duration of tuberculous activity in these patients ranged from two to twelve years. All of them represented treatment failures in spite of previous prolonged chemotherapy. In most cases, bacterial resistance to isoniazid or to streptomycin, or to both, was present. All of the patients were considered to have poor prognoses.

At the start of the study, the dosage employed was 0.5 gm. of neomycin sulfate dissolved in 2 to 3 ml. of distilled water to which was added 1 ml. of a solution of hyaluronidase[4] containing 150 U.S.P. units. Some of the patients received the medication by means of a nebulizer contained in the circuit of an intermittent positive pressure breathing apparatus; others, through a nebulizer connected to an ordinary oxygen tank. By the former method about twenty minutes were required to empty the nebulizer; and by the latter method, ten to fifteen minutes. An attempt to dissolve the neomycin in less fluid was found to prolong the period of time required for the administration, due to the greater viscosity of the resultant solution. The above dosage was given four times daily.

Twelve of the patients received neomycin and hyaluronidase. All developed increased cough within the first two weeks. In several patients the sputum became more tenacious, and the onset of moderate dyspnea was noted. The cough was particularly noticeable at night in a number of cases, interfering with sleep. These symptoms were sufficiently severe in all but 2 patients to require the cessation of treatment after only one to four weeks. Within two to five days after the aerosol was discontinued, the adverse symptoms subsided.

One patient tolerated the treatment for six weeks, but then complained of malaise, headache, and loss of appetite. One patient who noted only very slight bronchial irritation was able to continue the inhalations four times daily for twelve weeks, and twice

[1] Waksman, S. A., *et al.*: Am. Rev. Tubere., 1949, *60*, 78.

[2] Carr, D. T., *et al.*: Am. Rev. Tubere., 1951, *63*, 427.

[3] Written communication from Dr. Selman Waksman concerning an unpublished report received by him from G. Dumon, Mme. Dumon-Legre, and J. Taranger, 28 Rue Flegier, Marseille, France.

[4] Neomycin, as Mycifradin Sulfate, was supplied for this project through the courtesy of the Upjohn Company; and the hyaluronidase, as Wydase, was supplied through the courtesy of the Wyeth Laboratories.

daily for four additional weeks; but the treatment was then stopped because of the lack of improvement. One patient died during the course of therapy, but the aerosol treatments probably were not an important factor. Another patient with compensated cor pulmonale developed severe congestive heart failure, following an exacerbation of chronic bronchitis in which the irritant aerosol may have contributed.

A repetition of the treatment with neomycin and hyaluronidase, but with smaller doses and given at less frequent intervals, was attempted in 8 patients; but similar symptoms recurred usually within a few days. To exclude the possibility that the bronchial irritation was due to the hyaluronidase only, 2 patients new to the study were placed on neomycin in the same dosage without hyaluronidase. No signs of bronchial irritation were noted during a shortened clinical trial. One patient left the hospital without medical consent after only three weeks' treatment. In another the neomycin was discontinued after five weeks when slight albuminuria and hematuria were reported for the first time.

This comparatively favorable experience led to the next experiment. Seven of the patients who previously had not been able to tolerate the neomycin-hyaluronidase combination were given the aerosol of neomycin alone. Two patients were found unable to tolerate the drug more than once daily because of increased cough, and one of these also complained of nausea following the neomycin inhalations. One patient tolerated two treatments daily for ten weeks with only slight bronchial symptoms, but the therapy was then stopped because slight worsening was noted on roentgenographic examination. Another patient tolerated neomycin, 0.25 gm., in 2 ml. of distilled water twice daily for one week, but then left the hospital against advice. One had moderate headache, malaise, and anorexia after a few inhalations during a period of a week; another had marked wheezing and dyspnea after the second inhalation. One patient, however, has tolerated 0.25 gm. of neomycin with Alevaire® three times daily without toxicity for three months, and he has remained on this therapy.

Roentgenograms of the chest were taken monthly. No improvement was noted in any patient, and there was slight worsening in one. No sputum "conversions" were expected, and none were found by microscopy or culture. No patient gained weight. Urinalyses at weekly intervals showed slight albuminuria and hematuria in one patient who received neomycin without hyaluronidase for five weeks. This finding appeared after the fourth week and was no longer present one week after the treatment was stopped. Studies of the erythrocytes and leukocytes every two weeks, and various tests of liver function monthly, showed no abnormalities. Audiograms performed monthly revealed no hearing damage. Six patients developed black coating of the tongue between the first and third weeks of treatment. Nystatin orally for a few days relieved this condition, which did not reappear to any significant extent.

* * *

Thus, the present experience with neomycin administered by inhalation has been disappointing. Adverse symptoms, mainly increased severity of cough and the onset of dyspnea, have made it difficult to evaluate the therapeutic effect with regard to tuberculosis in most of the patients treated. When the treatment could be tolerated for any appreciable period of time, no objective or subjective benfits could be noted. However, it must be emphasized again that these patients were failures of previous treatment with agents of proved efficacy, whose tuberculosis was of long standing and whose prognosis was considered to be poor in each instance.

In so far as this experience permits forming an impression, it appears that the use of neomycin as an aerosol with or without hyaluronidase offers little promise of therapeutic effectiveness. Much undue discomfort may be expected when it is employed as a desperate

measure in "hopeless" patients with tuberculosis, such as those described. The value of this treatment in cases of recent tuberculous involvement could not be judged from the present experience. It is assumed that the advantage to be gained probably would not be commensurate with the side-effects likely to be encountered.

In summary: Neomycin and hyaluronidase were used as an aerosol in the treatment of 12 patients whose pulmonary tuberculosis had not been arrested by conventional measures. The aerosol treatment was found to be too irritating to the respiratory tract for prolonged use in all but one case. Neomycin without hyaluronidase was found to be only slightly less irritating. The duration of treatment varied from a few days to four months. No improvement in the tuberculosis was observed in any of the patients.

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[b] The writers wish to thank Dr. Selman Waksman, who was instrumental in providing them with the data of the French authors whose work they attempted in some measure to duplicate.

EFFECT OF KANAMYCIN ON MYCOBACTERIUM TUBERCULOSIS IN VITRO

Kanamycin is a new basic, water-soluble antimicrobial derived from *Streptomyces kanamyceticus*.[1] It appears to be effective against tubercle bacilli *in vitro* and has been shown to exert a marked suppressive effect on experimental tuberculosis in guinea pigs and mice.[2] It is the purpose of this report to confirm and extend the observations made by Yanagisawa and associates[2] through the use of a synthetic liquid medium with and without the presence of bovine albumin or human serum.

Virulent (H37Rv[3], Ravenel[3]) and attenuated (BCG[4]) strains of tubercle bacilli were used in these studies. The susceptibility of the organisms to kanamycin was determined in a modified Proskauer and Beck synthetic liquid medium.[5] When bovine albumin (fraction V, Armour) or normal human serum were used, they were sterilized by filtration and added

TABLE 1

SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS TO KANAMYCIN IN VITRO

Strain	Medium	Minimal Inhibitory Concentration (γ/ml.) Weeks of incubation			
		2	3	4	5
H37Rv	Basal	0.62	0.62	0.62	0.62
	Basal + albumin	1.25	1.25	2.5	5
	Basal + serum	0.62	1.25	2.5	5
Ravenel	Basal	0.62	0.62	0.62	0.62
	Basal + albumin	0.62	0.62	0.62	0.62
	Basal + serum	0.62	0.62	0.62	0.62
BCG	Basal + albumin	0.62	0.62	0.62	0.62
	Basal + serum	0.62	0.62	0.62	0.62

to the basal medium to make a final concentration of 0.5 per cent (albumin) or 5 per cent (serum). The medium was transferred aseptically in 5-ml. amounts to sterile screw-cap test tubes. Crystalline kanamycin sulfate[6] (Lot No. 26-27R-A9332) was dissolved in distilled water and sterilized by filtration through an ultrafine sintered glass filter. This sterile stock solution was then diluted serially so that, when added to the tubes of medium, the final concentrations of drug would be 0.62, 1.25, 2.5, 5, 10, 20, and 40 γ per ml. Control tubes received an equal volume of sterile distilled water. A portion of the pellicle from eight-day-old cultures of the test organisms grown on the basal medium were homogenized in saline and standardized[5] to contain 0.01 mg. per ml. The culture tubes were inoculated with 0.1 ml. (0.001 mg.) of bacterial suspension and incubated at 37°C. The amount of growth was recorded at weekly intervals.

[1] UMEZAWA, H., *et al.*: J. Antibiot., Ser. A, 1957, *10*.
[2] YANAGISAWA, K., *et al.*: In press.
[3] Obtained from Dr. William Steenken, Jr., Saranac Lake, New York.
[4] Obtained from Dr. S. R. Rosenthal, Tice Laboratory, Chicago, Illinois.
[5] YOUMANS, G. P., AND KARLSON, A. G.: Am. Rev. Tuberc., 1947, *55*, 529.
[6] Kindly supplied by Dr. A. R. Menotti, Bristol Laboratories Inc., Syracuse, New York.

The inhibitory effect of kanamycin on the three test organisms in the synthetic basal medium alone and in basal medium containing serum or albumin is presented in table 1. It will be seen that the drug completely inhibited growth of the Ravenel and BCG organisms throughout the full incubation period in a concentration of 0.62 γ per ml. regardless of the type of medium used. With the H37Rv strain, however, the minimal inhibitory concentration of kanamycin appeared to depend somewhat on the presence or absence of serum or albumin in the medium and on the incubation period. At a concentration of 0.62 γ per ml. in basal medium, growth of this strain was completely suppressed throughout five weeks of incubation; whereas, in the presence of bovine albumin or human serum, higher concentrations of drug were required for complete bacteriostasis, particularly after four to five weeks' incubation at 37°C. It remains to be seen whether this phenomenon represents emergence of resistant organisms, conversion of drug to an inactive form, or some other factor.

In summary: Under the experimental conditions described, kanamycin appeared to be highly active against *Mycobacterium tuberculosis in vitro*. With H37Rv (human) tubercle bacilli, the concentration of drug required for complete bacteriostasis was determined to some extent by the composition of the medium (presence or absence of albumin or serum) and the incubation period.

TUBERCULOSIS RESEARCH LABORATORY
VETERANS ADMINISTRATION HOSPITAL
WASHINGTON, D. C.

ROBERT A. PATNODE
PAUL C. HUDGINS

February 25, 1958

GIANT CELLS LINING HEALING TUBERCULOUS CAVITIES

The use of antimicrobial drugs, and particularly of isoniazid, has been accompanied by a significant increase of the so-called "open healing" of tuberculous cavities.[1-7] This phenomenon was decidedly rare before the antimicrobial era. Auerbach only found two cases of "open healing" in more than 2,000 autopsies of patients with chronic pulmonary tuberculosis when no antimicrobial drugs were given during life.[6] Pagel and Simmonds noticed that only five such cases had been reported up to 1942.[4] Medlar stated that he had never seen a completely healed open tuberculous cavity.[8] Wurm stated that the wall of a large tuberculous cavity would heal very rarely.[9]

At the present time, completely healed open tuberculous cavities are found in 5 to 10 per cent of surgical specimens, according to the available information. The walls of such cavities are entirely free from necrotic debris and are made of layers of lamellarly arranged and frequently hyalinized connective tissue. There is no epithelial membrane separating the fibrous tissue from the lumen except, in some cases, near the broncho-cavitary junction.[5, 10] One may observe, on the innermost collagenous layer, however, round or flat cells of probable mesenchymal origin.[2, 11] This view is corroborated by the finding of macroscopically "open-healed" tuberculous cavities lined by giant cells. Canetti, Thompson, Paillas, and their co-workers[3, 7, 12] have described groups of giant cells at the surface of healing, or macroscopically "open-healed" tuberculous cavities.

The present writers have recently found 4 macroscopically "open-healed" tuberculous cavities in one surgical specimen. Microscopically the cavity walls were composed either of fibrous connective tissue with no cellular lining or were bordered by flat, round, and atypical giant cells (figures 1–8). Most interesting were the two smallest ones, completely lined by atypical giant cells. In another case giant cells could be seen, almost in contact with the lumen of a cavity which had a soft necrotic border (figure 9).

Thus, three different aspects may be encountered, probably representing three successive phases of the same healing process; first, the giant cells are eliminated with the necrotic layer of the healing cavity; second, there is no necrotic debris, but flat, round, and giant cells are found lining the young connective tissue of the healing cavity wall; finally, these cellular elements disappear and the wall of the cavity is bare, and frequently hyalinized connective tissue is in immediate contact with the cavitary lumen.

The sterilization of the cavity wall probably depends upon the intensive destruction of bacilli, the softening of the caseous material, enhanced by isoniazid,[13] and the final

[1] Auerbach, O.: Am. Rev. Tuberc., 1955, *71*, 165.

[2] Poppe de Figueiredo, F., and De Paola, D.: Am. Rev. Tuberc., 1955, *71*, 186.

[3] Thompson, J. R.: Am. Rev. Tuberc., 1955, *72*, 601.

[4] Ibiapina, A., *et al.*: Med. Cirurg. Farm., 1954, *224*, 535.

[5] Corpe, R. F., and Stergus, I.: Am. Rev. Tuberc., 1957, *75*, 223.

[6] Auerbach, O., and Small, M. J.: Am. Rev. Tuberc., 1957, *75*, 242.

[7] Paillas, J., *et al.*: Rev. de la tuberc., 1957, *21*, 446.

[8] Medlar, E. M.: Am. Rev. Tuberc., March, 1955, *71* (Supplement).

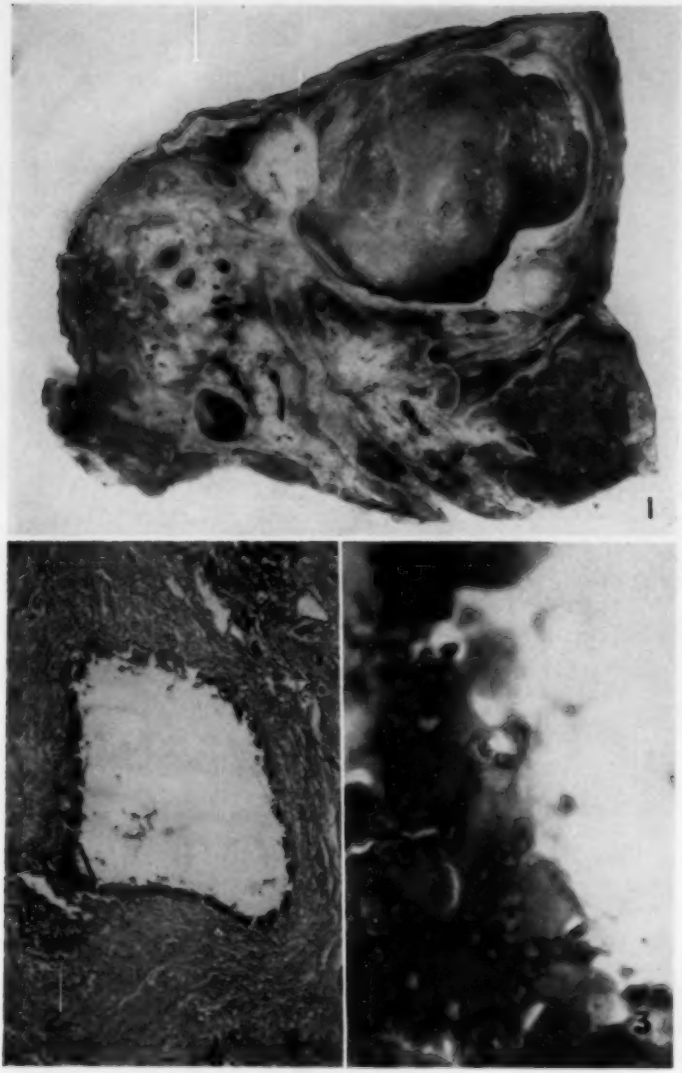
[9] Wurm, H.: in *Colapsoterapia de la Tuberculosis Pulmonar*, by Hein-Kremer-Schmidt, Editorial Labor, Buenos Aires, 1940.

[10] Auerbach, O., *et al.*: Am. Rev. Tuberc., 1953, *67*, 173.

[11] Purriel, C., *et al.*: Tórax, 1953, *2*, 115.

[12] Canetti, G.: Presse méd., 1956, *64*, 129.

[13] Curci, G.: Arch. tisiol., 1953, *8*, 206.

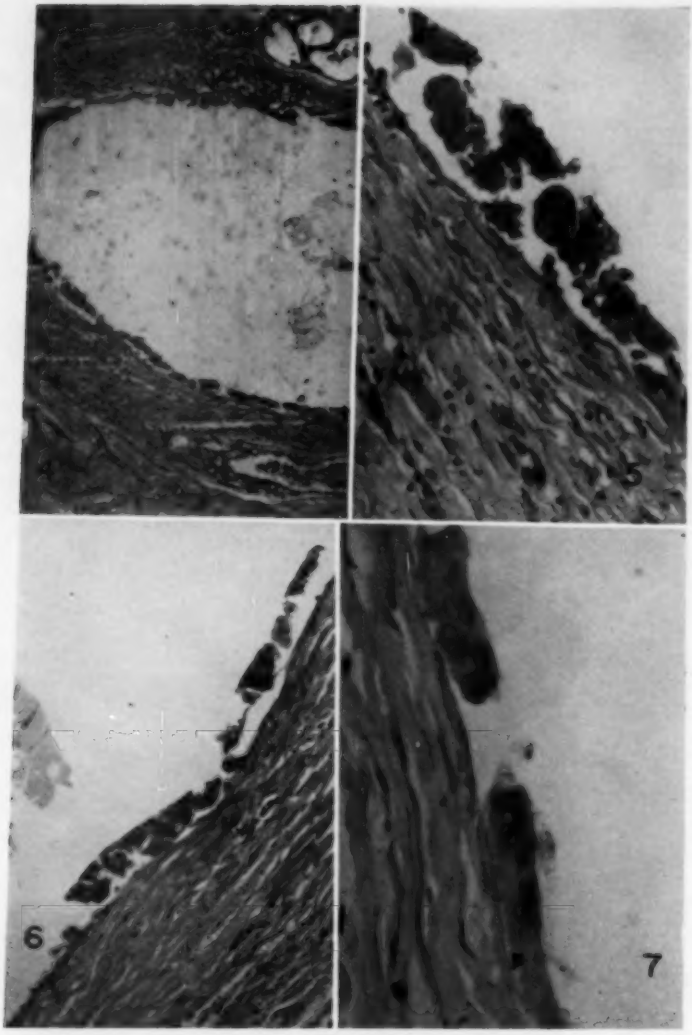


Patient A. M. P.

FIG. 1. Right lung of the patient (nine years old), showing macroscopically healed tuberculous cavities. Prolonged chemotherapy before the pneumonectomy.

FIG. 2. Small healing cavity lined by giant cells.

FIG. 3. A higher magnification of the field outlined in figure 2, showing a group of giant cells at the cavity surface.



Patient A. M. P.

FIG. 4. Another healing cavity lined by round and giant cells.

FIGS. 5, 6, and 7. Groups of giant cells, some of them very atypical, are seen at the surface of the largest cavities.

cleaning action of the macrophages, many of which are transformed into epithelioid and giant cells in disposing of the bacillary debris. A well-vascularized granulation tissue encircles the cavity wall and probably allows the achievement of adequate concentrations of the chemotherapeutic agents within the lumen of the cavity when the presence

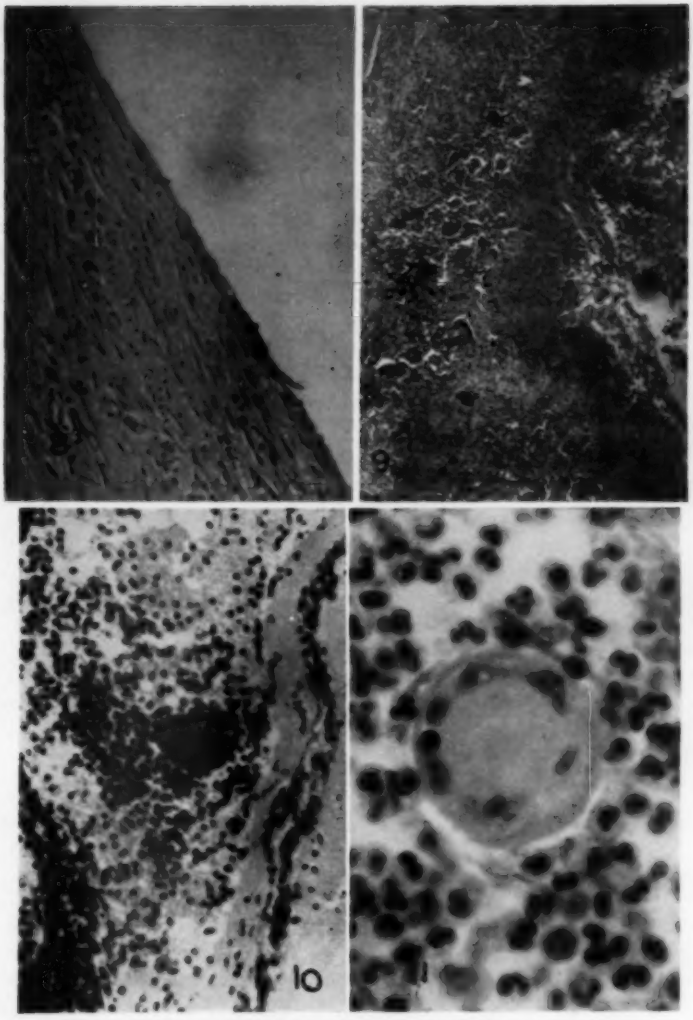


FIG. 8. *Patient A. M. P.* A completely healed stretch of cavitary wall.

FIG. 9. *Patient J. C. O.* Approximately two years of treatment with isoniazid and streptomycin. Several giant cells are separated from the cavitary lumen by a soft necrotic border.

FIG. 10. *Patient A. S.* Purulent sputum showing a giant cell. The patient was under prolonged chemotherapy.

FIG. 11. *Patient A. S.* A higher magnification, showing another giant cell in the sputum of the patient.

of the drugs is most necessary.[14] According to Russell and associates,[14] the elimination of the caseous material from the cavity would rid streptomycin and PAS of the blocking action of desoxyribonucleic acid and para-aminobenzoic acid. In such a manner, the more rapidly emerging isoniazid-resistant bacilli could be efficiently antagonized. Consequently, the sterilization of the cavity would coincide with the disappearance of necrotic debris and the formation of a bare fibrous capsule. An intervening phase presumably exists in which one might observe round and giant cells lining its innermost layer before the bacillary debris was totally destroyed. In figures 2–7 may be seen histologic aspects which are, in all probability, very transient findings. However, they may be important for clarifying the mechanisms involved in the healing of tuberculous cavities under the action of chemotherapeutic agents. On the other hand, according to the experience of the present writers, such cavities, as well as giant-cell pneumonia and evanescent granulomata[15] could explain the elimination of giant cells through the bronchial tree. The presence of these cells within bronchioles[2, 15] and in the sputum of tuberculous patients under long-term chemotherapy (figures 10 and 11) is a clear demonstration of such phenomenon.

Conjunto Sanatorial de Curicica and
Hospital-Sanatorio Santa Maria
Rio de Janeiro, Brazil

Flavio Poppe de Figueiredo
Domingos De Paola

March 10, 1958

[14] Russell, W. F., *et al.*: Am. Rev. Tuberc., 1955, *71*, 441.
[15] Poppe de Figueiredo, F., and De Paola, D.: Am. Rev. Tuberc., 1957, *76*, 871.

American Trudeau Society

Medical Section of the National Tuberculosis Association

THE ROLE OF BCG IN THE PREVENTION OF CLINICAL TUBERCULOSIS

A Statement of the Committee on Therapy

During the past ten years the perspective with regard to the prevention of tuberculosis has been influenced by the continued decline in tuberculosis morbidity and mortality, the advent of effective chemotherapy, the increased interest in the tuberculin test for case finding, and the availability of a standardized, lyophilized BCG vaccine. Today, as in the ATS statement of ten years ago, emphasis is placed on the importance of improving living conditions and general health as well as on educating the public and patients regarding the importance of case finding, early and adequate treatment of tuberculosis, and isolation of patients in the communicable phase of their disease.

The incidence of conversion to a positive tuberculin test is very low at the present time in the United States. However, in certain segments of the population where the incidence of tuberculosis is still high and the implementation of the above program is not feasible, BCG has a place. Suitable subjects for vaccination are tuberculin-negative individuals in the following groups:

1. Children living in areas with a high prevalence of tuberculosis among the younger age groups and certain adults living in highly special situations.
2. Physicians, nurses, medical and nursing students, laboratory workers, and hospital employees. If a hospital has established an adequate tuberculosis control program, very little exposure to tuberculosis will occur in that institution.
3. Persons unavoidably exposed to continued contact with infectious cases of tuberculosis in the home.
4. Patients, inmates, and employees of institutions such as mental hospitals and prisons, in which case-finding programs indicate that exposure to tuberculosis is likely to be high.

The protection from tuberculosis afforded by BCG vaccination is only relative and not permanent or entirely predictable. If the tuberculin test again becomes negative and the risk of infection continues, the BCG vaccination may need to be repeated.

BCG vaccination has certain disadvantages. Following vaccination the tuberculin skin test becomes positive. This eliminates an important diagnostic tool for the early detection of infection and may delay the institution of specific antituberculous chemotherapy. Mass BCG vaccination would be especially regrettable in areas of low prevalence of tuberculosis, since it would destroy the usefulness of the tuberculin skin test as a case-finding technique and make it virtually impossible to determine the incidence of new infections. BCG vaccination confers no benefit on persons already tuberculin positive and most cases of clinical tuberculosis may be expected to develop in this group, ineligible for vaccination.

Among nonreactors to tuberculin, there is the alternative of using BCG to further reduce the risk of clinical disease or of reserving the tuberculin test for making an early diagnosis, with subsequent treatment of the individual if desired. It is the responsibility of the physician to weigh the advantages and disadvantages in a particular situation.

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Obituaries

Henry Boswell

1884–1957

Henry Boswell, superintendent of the Mississippi State Sanatorium for the past forty years, died there on December 16, 1957. He was a state and national leader in medical and chest affairs throughout that time, and was a fine organizer and teacher.

Dr. Boswell obtained his medical degree at the University of Nashville Medical Department. He was on the faculty of the University of Mississippi for years and helped obtain a four-year Medical School.

Dr. Boswell was past president of the State Medical Association, the Southern Medical Association, and a member of the American College of Physicians. His greatest honors and labor came between 1928 and 1931 when he was sucessively president of the American Sanatorium Association (now the American Trudeau Society) and then of the National Tuberculosis Association. These offices alone are a testimonial to his ability and good works, but his staff and colleagues in Mississippi call him "wise leader, good friend, a great man, and guiding spirit."

He is survived by his widow, a son, four daughters, a brother, and fifteen grandchildren.

W. H. O., Jr.

Carl Puckett

1882–1957

Carl Puckett was born in Rogers, Arkansas, in 1882, the same year in which Koch isolated the tubercle bacillus. He lived to become the leading antituberculosis physician of Oklahoma, and died of a heart attack in Oklahoma City on September 27, 1957 at the age of seventy-four.

Dr. Puckett graduated in medicine from the St. Louis College of Physicians and Surgeons in 1905. He was urged to become a medical missionary but decided in favor of a crusading life against tuberculosis. It is said that an old clipping was found in his desk which exemplified his feeling: "For he that loveth God, loveth his brother also."

Dr. Puckett moved to Pryor, Oklahoma, worked in various private and civic medical posts, and became State Commissioner of Health in 1924. He became managing director of the Oklahoma Tuberculosis Association in 1927. He belonged to state and national public health associations, state and national Trudeau societies, and numerous other groups of public interest. He helped solve the problems of typhoid, malaria, and pellagra in his area. He was a pioneer in mass tuberculin testing, and prepared the antigen himself.

He is suvived by Mrs. Puckett, two daughters, and two grandchildren.

W. H. O., Jr.

Marguerite D. Shepard

1908–1957

Dr. Marguerite D. Shepard was born October 25, 1908, and died on April 29, 1957. She received her B.A. degree from Barnard College in 1931 and her M.D. from the New York University College of Medicine in 1937. She interned at Christ Hospital, Jersey City, New Jersey, and served as resident in tuberculosis at Belmont Hospital, Worcester, Massachusetts, and at Bellevue Hospital, New York, before taking charge of Cedarcrest in 1943.

She leaves many patients as well as family friends who will grieve her passing.

W. J. B.

E. E. Clovis

1879–1957

Dr. E. E. Clovis died May 13, 1957, at his home in Wheeling, West Virginia, after an illness of two months.

Doctor Clovis was born in Hebron, West Virginia, on August 27, 1879. He was a graduate of the College of Physicians and Surgeons of Baltimore, Maryland, and began the practice of medicine upon his graduation. In 1912, Dr. Clovis founded the Hopemont Sanitarium and was its superintendent from 1912 to 1924. For a number of years he was superintendent of the Ohio County Sanitarium. He was also the medical director for the Conservative Life Insurance Company.

Dr. Clovis was a Fellow of the American College of Physicians, of several other medical societies, and an honorary member of the West Virginia Tuberculosis and Health Association.

R. B. K.

William Brantingham Test

1902–1957

William B. Test came from a hospital administration family in Philadelphia, defeated his own personal tuberculosis problem, worked in the chest disease field for years, and died from a heart attack in Memphis, Tennesee, on May 25, 1957.

Dr. Test was born in 1902, was graduated in medicine from the University of Rochester, and was certified by the National Board. He was one of the last resident staff members of the old Desert Sanatorium in Tucson, and was once medical director of the Worcester County Sanatorium, Massachusetts. Dr. Test was finally a section chief in tuberculosis at the Veterans Administration Hospital in Memphis.

W. H. O., Jr.

Jacob W. Cutler

1900–1957

Jacob (Jack) W. Cutler of Philadelphia died in Wallingford, Pennsylvania, on August 31, 1957.

Dr. Cutler was a graduate of the University of Pennsylvania, was certified by the National Board of Medical Examiners and the American Board of Internal Medicine, and practiced as a chest specialist, to the advantage of his local area. He was director of the Delaware County Tuberculosis and Health Association, medical director of the Wawa Chest Hospital, and consultant of the Taylor Hospital and two Chester hospitals.

W. H. O., Jr.

John D. Cahill

1907–1957

John D. Cahill of New York, New York, was born April 30, 1907. He was graduated from Georgetown Medical School in 1932, and was a former director of Seton Hospital and St. Joseph's Hospital for Diseases of the Chest. He died in New York on September 13, 1957.

R. B. K.

Bret Ratner

1893–1957

Bret Ratner was born in Brooklyn, New York, was educated there, and received his medical degree from the University and Bellevue Hospital Medical College in 1918. Through the years his training included special work in pediatrics and allergy, and his name became one of the best known in those two specialties.

Dr. Ratner was a consultant in a dozen New York hospitals, a member of a number of specialty societies, and a faculty member of his own school. He was an honored speaker at international congresses and a member of the Nobel Prize Committee on Medicine. He had an enquiring mind and great energy. He published many articles and books, which included *Allergy in Relation to Pediatrics*. He died in New York Hospital of coronary occlusion on October 11, 1957, at the age of sixty-four.

W. H. O., Jr.

Charles Carthers Trembley

1873–1957

Charles C. Trembley lived and practiced in Saranac Lake during the age of its greatest function. He was a colleague of the associates and students of Edward L. Trudeau, was once director of the Saranac Lake Sanatorium, and was on the staff of the General Hospital. His medical education began at New York University Medical College. He died in Daytona Beach, Florida, on October 20, 1957, at the age of eighty-four years.

W. H. O., Jr.

Richard Charles Bernard

1903–1957

Richard Charles Bernard of Dallas, Texas, was born on November 12, 1903, in Hoboken, New Jersey. He was a graduate of the Academy of Medicine, Royal University of Florence, Italy, in 1929. He took an internship and residency in Bergen County Hospital, New Jersey, and served as assistant superintendent and medical director from 1935 to 1939. Dr. Bernard then moved to Texas, where he was on the staff of the McKnight Tuberculosis Sanatorium in San Angelo and of the Tuberculosis Clinic at the Cameron County Hospital in Harlingen. At the time of his death on December 29, 1957, he was on the staff of Woodlawn Tuberculosis Hospital, Dallas, Texas.

R. B. K.

Claude Gilbert Milham

1902–1957

Claude Gilbert Milham, a general practitioner but one of North Carolina's foremost chest specialists, died in October from a heart attack in Hamlet, North Carolina.

Dr. Milham was born in Boston in 1902. His family moved to Hamlet when he was seven. He was educated there, graduated from the University of North Carolina, and took his M.D. degree from Jefferson Medical College in Philadelphia. He worked in the state sanatorium and in two other sanatoriums from 1928 to 1934, after "curing" his own tuberculosis.

Dr. Milham enjoyed a life of civic service; helped found and direct Richmond and state tuberculosis associations; and was a member of several roentgenographic societies in addition to the medical associations. He is survived by his wife Agnes, two daughters,

and a brother and sister. His career would seem to represent the very fiber of antituberculosis work.

W. H. O., Jr.

John Albert Schindler

1903–1957

John Schindler was an amazing internist who was chief of medicine in a very good small-city clinic in Wisconsin, but who was interested in diseases of the chest and became a best-seller author of books on common-sense psychology. He lost his life in an automobile accident at the peak of his career.

Dr. Schindler was born in New Glarus, Wisconsin and received two degrees from the state university. His medical diploma was earned at Washington University of St. Louis. He soon helped found the Gnagi-Schindler Clinic (later the Monroe Clinic) in Monroe, Wisconsin. He was extremely well thought of by everyone who knew him, and occasionally took part in seminars and panels. One of these resulted in a lecture on radio; and his solid, homely, reassuring, philosophical discussion soon became a national vogue. He wrote several articles and two books, including *How to Live 365 Days a Year*. He quietly made one of the earliest roentgenographic case-finding surveys in private practice in 1935; it went unpublished but had considerable influence. He was a member of the American College of Physicians, the American Trudeau Society, and the College of Chest Physicians, and had been a member of the State Board of Medical Examiners.

Dr. Schindler is survived by his wife Dorothea, three daughters, and a brother and sister. It is hard to estimate his medical and social influence, but it was always top quality, beneficial, and very extensive.

W. H. O., Jr.

Frank I. Terrill

1901–1957

Frank I. Terrill died of a heart attack at Galen, Montana, where he was Superintendent of the Montana Tuberculosis Sanatorium at Deer Lodge. Dr. Terrill had cured his own lung disease in that institution after he graduated from the University of Michigan Medical School. He then dedicated his career, as did many others in that era, to use and improve the methods which might conquer the disease. He found the facilities in Montana scanty; worked to improve them; spent his vacations in study at modern clinics; and brought surgical methods to that remote area. He was a member of numerous medical and chest specialty groups, and leaves a host of grateful but sad Montanans.

W. H. O., Jr.

NOTICES

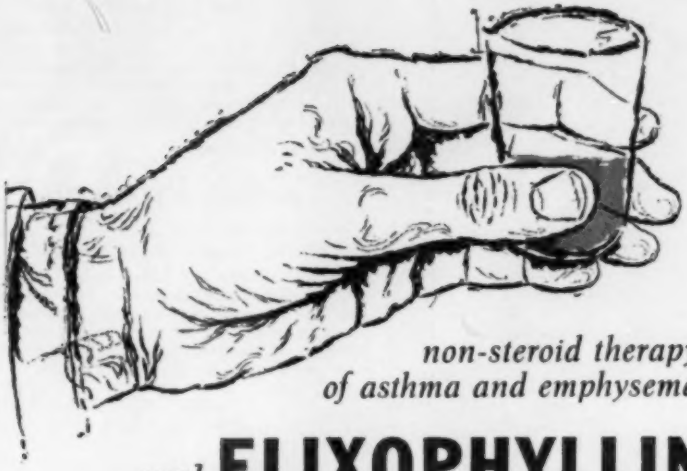
The following dates and locations of the next two annual meetings of the National Tuberculosis Association and the American Trudeau Society have been selected and approved:

1959—Chicago, Illinois—May 24–29
1960—Los Angeles, California—May 15–20

SYMPOSIUM ON PULMONARY DISEASES

The Eleventh Annual Symposium on Pulmonary Diseases will be held at Fitzsimons Army Hospital, Denver, Colorado, September 8–12th, 1958. This Symposium will be co-sponsored by the American Trudeau Society, the University of Colorado School of Medicine and Fitzsimons Army Hospital.

Information concerning enrollment may be obtained from the University of Colorado Medical School or from the Program Director, Colonel James A. Wier, Fitzsimons Army Hospital, Denver 8, Colorado.



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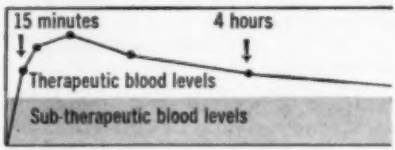

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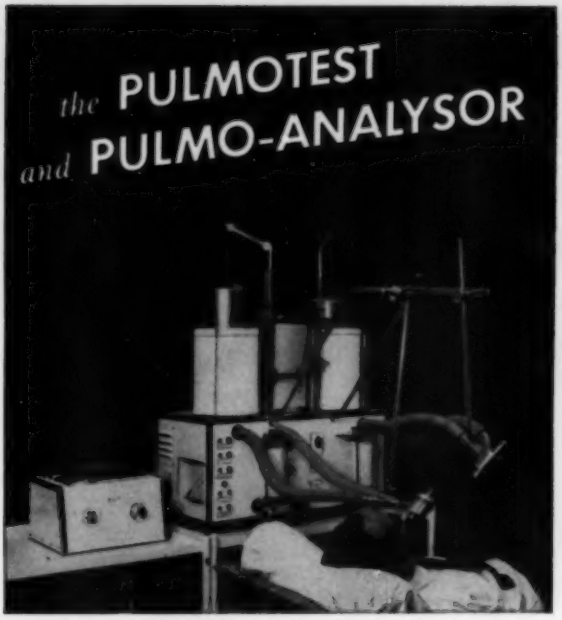

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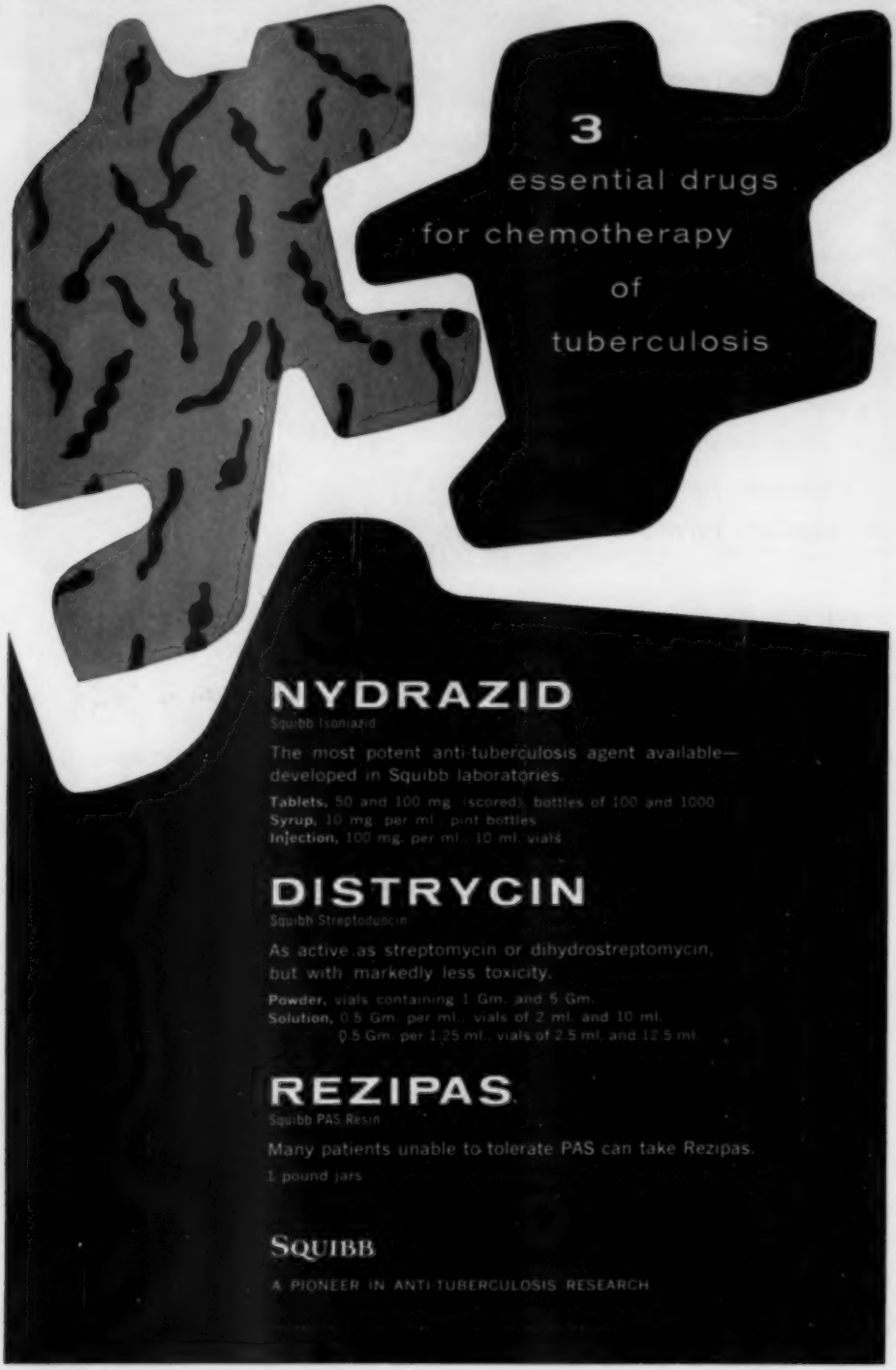
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